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(54) Title: **MAMMALIAN EDG-7 RECEPTOR HOMOLOGS**

(57) Abstract

The present invention is directed to nucleic acid sequence and amino acid sequences for mammalian EDG-7 receptor homologs, and particularly for human EDG-7 receptor homologs. The invention also provides methods for determining agonists and antagonists for EDG-7 receptors in addition to assays, expression vectors, host cells and methods for treating disorders associated with aberrant expression or activity of EDG-7.

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**MAMMALIAN EDG-7 RECEPTOR HOMOLOGS****FIELD OF THE INVENTION**

5        The present invention is in the field of molecular biology; more particularly, the present invention describes nucleic acid sequences and amino acid sequences for mammalian EDG-7 receptor homologs, and particularly for human EDG-7 receptor homologs.

**BACKGROUND OF THE INVENTION**

10      The family of edg receptors are commonly grouped with orphan receptors because their endogenous ligands are not known (for example see Hla T and Maciag T (1990) J Biol. Chem. 265:9308-13 ; US 5,585,476). Recently, however, lysophosphatidic acid has been demonstrated to be the endogenous ligand for the edg-2 receptor (Hecht et al. (1996) J. Cell. 15      Biol. 135: 1071-1083; An et al. (1997) Biochem. Biophys. Res. Comm. 213: 619-622).

20      The edg family of receptors is seven transmembrane G protein coupled receptors (T7Gs or GPCRs). T7Gs are so named because of their seven hydrophobic domains that span the plasma membrane and form a bundle of antiparallel  $\alpha$  helices. These transmembrane segments (TMS) are designated by roman numerals I-VII and account for structural and functional features of the receptor. In most cases, the bundle of helices forms a binding pocket; however, when the binding site must accommodate more bulky molecules, the extracellular N-terminal segment or one or more of the three extracellular loops participate in binding and in subsequent induction of conformational change in intracellular portions of the 25      receptor. Specifically: the TM-VII is generally a highly conserved portion of the T7G receptors, and is often critically involved in ligand binding and receptor activation; the intracellular carboxy-terminal is involved in interactions with intracellular proteins, including those that transduce intracellular signals upon receptor activations; the carboxy-terminal is usually hydrophilic and highly antigenic relative to the receptor polypeptide as a whole and 30      shows greatly reduced conservation.

Once the receptor is activated, the receptor, in turn, interacts with an intracellular G-protein complex which mediates further intracellular signaling activities, including: generally,

the production of second messengers such as cyclic AMP (cAMP), phospholipase C, inositol triphosphate; activation of protein kinases; and alteration in the expression of specific genes.

T7G receptors are expressed and activated during numerous developmental and  
5 disease processes. Identification of a novel T7G receptor provides the opportunity to diagnose or intervene in such processes, and the receptor can be used in screening assays to identify physiological or pharmaceutical molecules which trigger, prolong or inhibit its activity or differentially modulate distinct intracellular pathways which are controlled from T7G receptors.

10

## SUMMARY OF THE INVENTION

The invention provides isolated and unique nucleotide sequences that encode novel mammalian EDG-7 receptor homologs, and particularly, novel human EDG-7 (HEDG-7)  
15 receptor homologs. Herein, the nucleotide sequence encoding HEDG-7 is designated hedg-7.

The present invention also relates to the isolated and unique nucleotide sequences of the complement of hedg-7 mRNA. In addition, the invention features nucleotide sequences, which hybridize under stringent conditions to hedg-7.

20

The present invention also relates to nucleotide sequences that encode fragments or portions of hedg-7, or complements thereof, in addition to expression vectors and host cells comprising such nucleotide sequences.

25

The present invention also provides amino acid fragments, particularly fragments in the TM-VII and carboxy-terminal domains that are useful as antibodies for HEDG-7.

Furthermore, the invention relates to the use of the nucleotide sequences of hedg-7 and the amino acid sequences of HEDG-7, or its variants, in the diagnosis or treatment of

30 diseased cells and/or tissues associated with aberrant expression of hedg-7.

Additional aspects of the invention include the antisense DNA of hedg-7; cloning or expression vectors containing hedg-7; host cells or organisms transformed with expression

vectors containing hedg-7; chromosomal localization of hedg-7; expression and tissue distribution of hedg-7; a method for the production and recovery of purified HEDG-7 from host cells; purified protein, HEDG-7, which can be used to identify inhibitors for the downregulation of signal transduction involving HEDG-7; and methods of screening for 5 ligands of hedg-7 using transformed cells.

In particular, the present invention provides an isolated nucleotide sequence selected from the group consisting of:

- (a) the nucleotide sequence comprising nucleotides 16- 1170 of Figure 1A (SEQ ID 10 NO:1);
- (b) the nucleotide sequence comprising nucleotides 13-1167 of Figure 1B (SEQ ID NO:2);
- (c) a nucleotide sequence with 70% sequence identity to (a) or (b), more preferably at least about 80-85% sequence identity, and even more preferably at least about 90% sequence 15 identity, and most preferably at least about 95% sequence identity, and which nucleotide sequence hybridizes under stringent conditions to the nucleotide sequence of (a) or (b), respectively, or portions thereof;
- (d) a nucleotide sequence which encodes the amino acid sequence of Figure 2A (SEQ ID NO:3); and
- (e) a nucleotide sequence which encodes the amino acid sequence of Figure 2B (SEQ 20 ID NO:4).

There is also provided: expression vectors; host cells; purified amino acid sequences; complementary nucleic acid sequences; biologically active fragments; and hybridization 25 probes, for such nucleotide sequences and their encoded amino acid sequences.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A (SEQ ID NO:1) shows a nucleotide sequence of hedg-7, nucleotides 16-1170 of 30 which encode full length HEDG-7, derived from BAC and PAC clones.

Figure 1B (SEQ ID NO:2) shows a nucleotide sequence of hedg-7, nucleotides 13-1167 of which encode full length HEDG-7, derived from the pc3-hedg7#M10 clone.

Figure 2A (SEQ ID NO:3) shows the predicted amino acid sequence encoded by hedg-7 of Figure 1A.

5 Figure 2B (SEQ ID NO:4) shows the amino acid sequence encoded by hedg-7 of Figure 1B.

Figure 3A shows the alignment of the nucleotide sequence of hedg-7 of Figure 1A aligned with the amino acid sequence of HEDG-7 of Figure 2A.

10 Figure 3B shows the alignment of the amino acid sequences of Figure 2A and Figure 2B. There are two amino acid substitutions relative to the HEDG-7 amino acid sequence of Figure 2A found at positions 140 and 378.

15 Figure 4 shows an alignment of the HEDG-7 predicted amino acid sequence of Figure 2A with the amino acid sequences of other EDG receptors.

Figure 5 shows a partial genomic nucleotide sequence of rat edg-7.

20 Figure 6 shows a predicted partial amino acid sequence of rat HEDG-7

Figure 7 is a bar graph showing the SRE response of a pcDNA3-HEDG7 clone to 10  $\mu$ M S1P.

25 Figure 8 is a bar graph showing the SRE response of 5 $\mu$ M SPC, S1P, LPA, lysophosphatidylcholine (LPC), edelfosine, psychosine, anandamide or 2-arachidonoylglycerol. .

Figure 9 is a graph showing the SRE dose response of of S1P, SPC, psychosine, glucopsychosine and dihydrosphingosine 1-phosphate (dihydro-S1P).

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## DETAILED DESCRIPTION OF THE INVENTION

The invention relates in one respect to polynucleotides, in their isolated form, that encode the human edg-7 receptor. The EDG receptors are characterized by structural features common to the G-protein coupled receptor class, including seven transmembrane regions, and by the functional properties of binding lysophingolipids selectively. When expressed functionally in a host cell, i.e., in operable linkage with a responsive second messenger system the EDG-7 receptors are capable further of responding to lysophingolipid or binding by signal transduction. In this regard, the activity of HEDG-7 receptor can be measured using any of a variety of appropriate functional assays described hereinbelow.

As used herein and designated by the upper case abbreviation, HEDG-7, refers to a human EDG-7 receptor homolog in either naturally occurring or synthetic form. The HEDG-7 receptor is activated by S1P and SPC and includes the amino acid sequence of Figure 2A or 2B and biologically active fragments thereof. More particularly, the HEDG-7 receptors preferably have at least 90% sequence identity with each other, and more preferably at least 95% sequence identity with each other.

All publications and patent applications mentioned herein are incorporated by reference for the purpose of describing the methodologies, cell lines and vectors, among other things. However, nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure, for example, by virtue of prior invention.

Definitions

25

The following definitions are used herein for the purpose of describing particular terms used in the application. Any terms not specifically defined should be given the meaning commonly understood by one of ordinary skill in the art to which the invention pertains.

30

As used herein "isolated" means separated from nucleotide sequences that encode other proteins. In the context of polynucleotide libraries, for instance, a hedg-7 receptor-encoding nucleotide sequence is considered "isolated" when it has been selected, and hence

removed from association with other nucleotide sequences within the library. Such nucleotide sequences may be in the form of RNA, or in the form of DNA including cDNA, genomic DNA and synthetic DNA.

5 As used herein "purified" refers to sequences that are removed from their natural environment, and are isolated or separated, and are at least 60% free, preferably 75 % free, and most preferably 90% free from other components with which they are naturally associated.

10 An "oligonucleotide" is a stretch of nucleotide residues, which has a sufficient number of bases to be used as an oligomer, amplimer or probe in a polymerase chain reaction (PCR). Oligonucleotides are prepared from genomic or cDNA sequence and are used to amplify, reveal or confirm the presence of a similar DNA or RNA in a particular cell or tissue. Oligonucleotides or oligomers comprise portions of a DNA sequence having at least 15 about 10 nucleotides and as many as about 35 nucleotides, preferably about 25 nucleotides.

"Probes" may be derived from naturally occurring, recombinant, or chemically synthesized single - or double - stranded nucleic acids or be chemically synthesized. They are useful in detecting the presence of identical or similar sequences.

20 A "portion" or "fragment" of a nucleotide or nucleic acid sequence comprises all or any part of the sequence having fewer nucleotides than about 6 kb, preferably fewer than about 1 kb. A portion or fragment can be used as a probe. Such probes may be labeled with reporter molecules using nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. To optimize reaction conditions and to eliminate false positives, nucleic acid probes may be used in Southern, Northern or in situ hybridizations to determine whether 25 DNA or RNA encoding HEDG-7 is present in a cell type, tissue, or organ.

30 "Reporter" molecules are those radionuclides , enzymes, fluorescent, chemiluminescent, or chromogenic agents which associate with, establish the presence of, and may allow quantification of a particular nucleotide or amino acid sequence.

“Recombinant nucleotide variants” encoding HEDG-7 may be synthesized by making use of the “redundancy” in the genetic code. Various codon substitutions, such as the silent changes which produce specific restriction sites or codon usage-specific mutations, may be introduced to optimize cloning into a plasmid or viral vector or expression in a particular 5 prokaryotic or eukaryotic host system, respectively.

“Chimeric” molecules may be constructed by introducing all or part of the nucleotide sequence of this invention into a vector containing additional nucleic acid sequence which might be expected to change any one (or more than one) of the following HEDG-7 10 characteristics: cellular location, distribution, ligand-binding affinities, interchain affinities, degradation/turnover rate, signaling, etc.

“Biologically Active or Active” refers to those forms, fragments, or domains of any HEDG-7 polypeptide which retain at least some of the biological and/or antigenic activities 15 of any naturally occurring HEDG-7.

“Naturally occurring HEDG-7” refers to a polypeptide produced by cells which have not been genetically engineered and specifically contemplates various polypeptides arising from post-translational modifications of the polypeptide including but not limited to 20 acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

“Derivative” refers to those amino acid sequences and nucleotide sequences which have been chemically modified. Such techniques for polypeptide derivatives include: ubiquitination; labeling (see above); pegylation (derivatization with polyethylene glycol); and 25 chemical insertion or substitution of amino acids such as ornithine which do not normally occur in human proteins. A nucleotide sequence derivative would encode the amino acid which retains its essential biological characteristics of the natural molecule.

“Recombinant polypeptide variant” refers to any polypeptide which differs from 30 naturally occurring HEDG-7 by amino acid insertions, deletions and/or substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest may be found by

comparing the sequence of HEDG-7 with that of related polypeptides and minimizing the number of amino acid sequence changes made in highly conserved regions.

Amino acid "substitutions" are conservative in nature when they result from replacing 5 one amino acid with another having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine.

"Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The 10 variation allowed may be experimentally determined by producing the peptide synthetically or by systematically making insertions, deletions, or substitutions of nucleotides in the hedg-7 sequence using recombinant DNA techniques.

A "signal or leader sequence" can be used, when desired, to direct the polypeptide 15 through a membrane of a cell. Such a sequence may be naturally present on the polypeptides of the present invention or provided from heterologous sources by recombinant DNA techniques.

An "oligopeptide" is a short stretch of amino acid residues and may be expressed from 20 an oligonucleotide. It may be functionally equivalent to and the same length as (or considerably shorter than) a "fragment", "portion", or "segment" of a polypeptide. Such sequences comprise a stretch of amino acid residues of at least about 5 amino acids and often about 17 or more amino acids, typically at least about 9 to 13 amino acids, and of sufficient length to display biological and/or antigenic activity.

25

"Inhibitor" is any substance which retards or prevents a biochemical, cellular or physiological reaction or response. Common inhibitors include but are not limited to antisense molecules, antibodies, and antagonists.

30 "Standard" is a quantitative or qualitative measurement for comparison. It is based on a statistically appropriate number of normal samples and is created to use as a basis of comparison when performing diagnostic assays, running clinical trials, or following patient treatment profiles.

“Stringent conditions” is used herein to mean conditions that allow for hybridization of substantially related nucleic acid sequences. Such hybridization conditions are described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, 1989. Generally, stringency occurs within a range from about 5 °C below the melting temperature of the probe to about 20 °C – 25 °C below the melting temperature. As understood by ordinary skilled persons in the art, the stringency conditions may be altered in order to identify or detect identical or related nucleotide sequences. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in solution or immobilization, etc.) and the concentration of the salts and other components (e.g. the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency.

15 “Animal” as used herein may be defined to include human, domestic (cats dogs, etc.), agricultural (cows, horses, sheep, etc.) or test species (mouse, rat, rabbit, etc.).

20 “Nucleotide sequences” as used herein are oligonucleotides, polynucleotides, and fragments or portions thereof, and are DNA or RNA of genomic or synthetic origin which may be single or double stranded, and represent the sense or complement or antisense strands.

25 “Sequence Identity” is known in the art, and is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences, particularly, as determined by the match between strings of such sequences. Sequence identity can be readily calculated by known methods (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two sequences, the term is well known to skilled artisans (see, for example, *Sequence Analysis in Molecular Biology*; *Sequence Analysis Primer*; and Carillo, H., and Lipman, D.,

SIAM *J. Applied Math.*, 48: 1073 (1988)). Methods commonly employed to determine identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM *J. Applied Math.*, 48: 1073 (1988) or, preferably, in Needleman and Wunsch, *J. Mol. Biol.*, 48: 443-445, 1970, wherein the parameters are as set in version 2 of  
5 DNASIS (Hitachi Software Engineering Co., San Bruno, CA). Computer programs for determining identity are publicly available. Preferred computer program methods to determine identity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *Nucleic Acids Research* 12(1): 387 (1984)), BLASTP,  
BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol.* 215: 403-410 (1990)). The  
10 BLASTX program is publicly available from NCBI ([blast@ncbi.nlm.nih.gov](mailto:blast@ncbi.nlm.nih.gov)) and other sources (*BLAST Manual*, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894;  
Altschul, S., et al., *J. Mol. Bio.* 215: 403-410 (1990)). Computational Molecular Biology,  
Lesk, A.M, ed. Unless specified otherwise in the claims, the percent identity for the purpose  
of interpreting the claims shall be calculated by the Needleman and Wucnsch algorithm with  
15 the parameters set in version 2 of DNASIS.

Phospholipids have been demonstrated to be important regulators of cell activity, including mitogenesis (Xu et al. (1995) *J. Cell. Physiol.*, 163: 441-450) and apoptosis, cell adhesion and regulation of gene expression. Specifically, for example, LPA elicits growth  
20 factor-like effects on cell proliferation (Moolenaar (1996) *J. Biol. Chem.*, 270: 12949-12952) and cell migration (Imamura et al. (1993) *Biochem. Biophys. Res. Comm.*, 193: 497-503). Further, considerable circumstantial evidence indicates that phospholipids may be involved in various disease states including cancer (Imamura et al., (1993) *Biochem. Biophys. Res. Comm.*, 193: 497-503); diseases having an inflammatory component (Fourcade et al. (1995),  
25 *Cell*, 80(6): 919-927, including adult respiratory distress, neurodegeneration (Jalink et al. (1993) *Cell Growth Differ.*, 4: 247-255), rheumatoid arthritis (Natiarajan et al. (1995) *J. Lipid Res.*, 36(9): 2005-2016), psoriasis and inflammatory bowel disease. Thus, modulators of HEDG-7 expression or activity is likely to be useful in treatment or prevention of such disease states.

30

The edg receptor family of T7G receptors has been subdivided into 2 subgroups on the basis of sequence similarity and genomic organization (Chun, Contos & Munroe, in press). We have determined that edg-2, edg-5 (U.S. S.N. 08/997,803) and edg-6 (Genbank

Accession AF011466) respond to LPA as an agonist, and share a common intron structure within their coding regions. Edg-1, edg-3 and edg-4/H218 (Accession U10699) have intronless coding regions and respond to S1P and SPC as agonists. The present T7G receptor, HEDG-7, has no intron within the coding region; however, its amino acid sequence 5 shows motifs characteristic of both edg family subgroups. (See Figure 4)

One aspect of the present invention is a method for using recombinant HEDG-7 receptors in an assay for screening ligands and potential drug candidates. Although the use of T7G receptors in high-throughput screening is well-known, no such screen has been reported 10 for the HEDG-7 receptor. More specifically, the novel HEDG-7 receptor presented herein can be used to identify and rank the relative potency and efficacy of potential agonists. These compounds may be useful inasmuch as they would be expected to modulate cellular or physiological responses to HEDG-7 agonists, or to initiate or supplement HEDG-7 signaling in cells where the receptor occurs. Equally, once a quantitative and reliable assay is 15 established, it can readily be applied to identify and rank the relative potency and efficacy of receptor antagonists. This application, without limiting other aspects, of the screening methods described herein is specifically contemplated and incorporated within the scope of this invention.

It was determined that S1P and SPC are agonists for HEDG-7. (See Figures 7-9) On 20 the other hand, it was determined that LPA, edelfosine, psychosine, glucosychosine, dihydro-S1P, anadamide and 2-arachidonylglycerol do not act as HEDG-7 agonists. (See Figures 8,9)

Other HEDG-7 ligands are likely to be found among the phospholipid class of 25 compounds. Therefore, in one embodiment, phospholipid molecules could be screened to identify ligands. Particularly, it is believed that potential ligands include fatty acid chains of differing length, such as 16, 17, 18, 19, 20, 22 and 24 carbon units, with or without 1, 2, 3 or 4 unsaturated carbon-carbon bonds. Phosphatidic acid, sphingosine, numerous lysophospholipids and lysosphingolipids play roles in pathophysiology of human diseases. 30 HEDG-7, by responding to low circulating or locally produced levels of such bioactive lipids, may initiate significant components of the pathophysiological response in such a disease. Edg-7 was not detected on a multi-tissue Northern blot, suggesting that high-level expression is not widespread in normal tissues. RT-PCR has demonstrated the presence in edg-7 mRNA

in mammary gland and lymph node tissue. Further, RT-PCR, has demonstrated edg-7 RNA expression in several rat tissues, including colon, lung, spleen, hypothalamus, hindbrain, small intestine, liver and kidney. Edg-7 cDNA inserts have also been detected in human cDNA libraries synthesized from small intestine and fetal brain and may be expressed within 5 specialized cell populations of these and other tissues.

The nucleotide sequences encoding HEDG-7 (or their complement) have numerous applications in techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use in the construction of oligomers for PCR, 10 use for chromosome and gene mapping, use in the recombinant production of HEDG-7, and use in generation of antisense DNA or RNA, their chemical analogs and the like. Uses of nucleotides encoding HEDG-7 disclosed herein are exemplary of known techniques and are not intended to limit their use in any technique known to a person of ordinary skill in the art. Furthermore, the nucleotide sequences disclosed herein may be used in molecular biology 15 techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, e.g., the triplet genetic code, specific base pair interactions, etc.

It will be appreciated by those skilled in the art that as a result of the degeneracy 20 of the genetic code, a multitude of HEDG-7 encoding nucleotide sequences may be produced. Some of these will only bear minimal homology to the nucleotide sequence of the known and naturally occurring HEDG-7. The invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based 25 on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring hedg-7, and all such variations are to be considered as being specifically disclosed.

Although the nucleotide sequences which encode HEDG-7, its derivatives or its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally 30 occurring hedg-7 under stringent conditions, it may be advantageous to produce nucleotide sequences encoding HEDG-7 or its derivatives possessing a substantially different codon usage. Codons can be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic expression host in accordance with the frequency

with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding HEDG-7 and/or its derivatives without altering the encoded amino acid sequence include the production of RNA transcripts having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring

5 sequence.

Human genes often show considerable actual polymorphism; that is, variation in nucleotide sequence among a fraction of the entire human population. In many cases this polymorphism can result in one or more amino acid substitutions. While some of these  
10 substitutions show no demonstrable change in function of the protein, others may produce varying degrees of functional effects. In fact, many natural or artificially produced mutations can lead to expressible HEDG proteins. Each of these variants, whether naturally or artificially produced, is considered to be equivalent and specifically incorporated into the present invention.

15

Nucleotide sequences encoding HEDG-7 may be joined to a variety of other nucleotide sequences by means of well established recombinant DNA techniques (Sambrook J et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor NY; or Ausubel FM et al (1989) Current Protocols in Molecular Biology, 20 John Wiley & Sons, New York City). Useful nucleotide sequences for joining to hedg-7 include an assortment of cloning vectors such as plasmids, cosmids, lambda phage derivatives, phagemids, and the like. Vectors of interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, etc. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease sensitive sites, and selectable markers for one or more host cell 25 systems.

Another aspect of the subject invention is to provide for hedg-7 specific hybridization probes capable of hybridizing with naturally occurring nucleotide sequences encoding 30 HEDG-7. Such probes may also be used for the detection of similar T7G encoding sequences and should preferably contain at least 60% nucleotide identity to hedg-7 sequence . The hybridization probes of the subject invention may be derived from the nucleotide sequence presented in the figures for hedg-7 or from genomic sequences including promoter,

enhancers, introns or 3'-untranslated regions of the native gene. Hybridization probes may be labeled by a variety of reporter molecules using techniques well known in the art. Preferably, the hybridization probes incorporate at least 15 nucleotides, and preferably at least 25 nucleotides, of the hedg-5 receptor. Suitable hybridization probes would include: consensus 5 fragments, for example, those regions of the rat and human edg-5 receptor that are identical; the extracellular edg-7 binding domain; the stipulated transmembrane regions and the C-terminal portion of the receptor.

It will be recognized that many deletional or mutational analogs of nucleic acid 10 sequences for HEDG-7 will be effective hybridization probes for HEDG-7 nucleic acid. Accordingly, the invention relates to nucleic acid sequences that hybridize with such HEDG-7 encoding nucleic acid sequences under stringent conditions.

Stringent conditions will generally allow hybridization of sequence with at least about 15 70% sequence identity, more preferably at least about 80-85% sequence identity, even more preferably at least about 90% sequence identity, and most preferably with at least about 95% sequence identity. Hybridization conditions and probes can be adjusted in well-characterized ways to achieve selective hybridization of human-derived probes. Nucleic acid molecules that will hybridize to HEDG-7 encoding nucleic acid under stringent conditions can be 20 identified functionally, using methods outlined above, or by using for example the hybridization rules reviewed in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, 1989. Without limitation, examples of the uses for hybridization probes include: histochemical uses such as identifying tissues that express HEDG-7; measuring mRNA levels, for instance to identify a sample's tissue type or to 25 identify cells that express abnormal levels of HEDG-7; and detecting polymorphisms in the HEDG-7. RNA hybridization procedures are described in Maniatis et al. Molecular Cloning, a Laboratory Manual (Cold Spring Harbor Press, 1989). PCR as described US Patent No's. 4,683,195; 4,800,195; and 4,965,188 provides additional uses for oligonucleotides based upon the nucleotide sequence which encodes the EDG-7 sequences of the invention. Such 30 probes used in PCR may be of recombinant origin, chemically synthesized, or a mixture of both. Oligomers may comprise discrete nucleotide sequences employed under optimized conditions for identification of hedg-7 in specific tissues or diagnostic use. The same two oligomers, a nested set of oligomers, or even a degenerate pool of oligomers may be

employed under less stringent conditions for identification of closely related DNA's or RNA's. Rules for designing PCR primers are now established, as reviewed by PCR Protocols, Cold Spring Harbor Press, 1991. Degenerate primers, i.e., preparations of primers that are heterogeneous at given sequence locations, can be designed to amplify nucleic acid sequences that are highly homologous to, but not identical to hedg-7. Strategies are now available that allow for only one of the primers to be required to specifically hybridize with a known sequence. See, Froman et al., Proc. Natl. Acad. Sci. USA 85: 8998, 1988 and Loh et al., Science 243: 217, 1989. For example, appropriate nucleic acid primers can be ligated to the nucleic acid sought to be amplified to provide the hybridization partner for one of the primers. In this way, only one of the primers need be based on the sequence of the nucleic acid sought to be amplified. PCR methods of amplifying nucleic acid will utilize at least two primers. One of these primers will be capable of hybridizing to a first strand of the nucleic acid to be amplified and of priming enzyme-driven nucleic acid synthesis in a first direction. The other will be capable of hybridizing the reciprocal sequence of the first strand (if the sequence to be amplified is single stranded, this sequence will initially be hypothetical, but will be synthesized in the first amplification cycle) and of priming nucleic acid synthesis from that strand in the direction opposite the first direction and towards the site of hybridization for the first primer. Conditions for conducting such amplifications, particularly under preferred stringent hybridization conditions, are well known. See, for example, PCR Protocols, Cold Spring Harbor Press, 1991.

Other means of producing specific hybridization probes for hedg-7 include the cloning of nucleic acid sequences encoding HEDG-7 or HEDG-7 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate reporter molecules.

More particularly, a method for detection of polynucleotides that hybridize with hedg-7 is exemplified in Example 9, wherein a positive test correlates to approximately at least 70 % identitiy, and more preferably at least 80-85% sequence identity.

It is possible to produce a DNA sequence, or portions thereof, entirely by synthetic chemistry. After synthesis, the nucleic acid sequence can be inserted into any of the many available DNA vectors and their respective host cells using techniques which are well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into the  
5 nucleotide sequence. Alternately, a portion of sequence in which a mutation is desired can be synthesized and recombined with longer portion of an existing genomic or recombinant sequence.

The nucleotide sequence for hedg-7 can be used in an assay to detect inflammation or  
10 disease associated with abnormal levels of HEDG-7 expression. The cDNA can be labeled by methods known in the art, added to a fluid, cell or tissue sample from a patient, and incubated under hybridizing conditions. After an incubation period, the sample is washed with a compatible fluid which optionally contains a reporter molecule. After the compatible fluid is rinsed off, the reporter molecule is quantitated and compared with a standard as  
15 previously defined.

A diagnostic test for aberrant expression of HEDG-7 can accelerate diagnosis and proper treatment of abnormal conditions of for example, the heart, kidney, lung and testis. Specific examples of conditions in which aberrant expression of HEDG-7 may play a role  
20 include adult respiratory distress, asthma, rheumatoid arthritis, cardiac ischemia, acute pancreatitis, septic shock, psoriasis, acute cyclosporine nephrotoxicity and early diabetic glomerulopathy, as well as lung damage following exposure to cigarette smoke, asbestos or silica.

25 New nucleotide sequences can be assigned to chromosomal subregions by physical mapping. The mapping of new genes or nucleotide sequences provide useful landmarks for investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a disease or syndrome, such as ataxia telangiectasia (AT), has been crudely localized by genetic linkage to a particular genomic region, for example, AT to 1 1q22-23  
30 (Gatti et al (1988) Nature 336:577-580), any sequences mapping to that area may represent or reveal genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in gene sequence between normal and carrier or affected individuals.

The chromosomal localization of the hedg-7 gene was mapped to chromosome 19p13.3 by fluorescent in situ hybridization (FISH). This localization is nearly identical to the localizations of edg-6 and edg-4, as determined via FISH analysis with appropriate BAC or 5 PAC DNA clones. Although the precise distances and relative orders of these three genes have not yet been determined, it raises the possibility of an edg gene cluster that may be maintained by evolutionary selection.

A search of Genbank with the genomic sequence of hedg-7 revealed a 94.7% identical 10 sequence over 339 bp of the 3' flanking region of the genomic hedg-7 to the Genbank entry HSRTLIPE (accession X65642). The HSRTLIPE sequence was identified as a repetitive dinucleotide element within a cosmid clone (26710) from chromosome 19p13.1-19p13.2, containing a portion of the gene for hormone-sensitive lipase LIPE (Levitt et al, Cytogenet Cell Genet 1995;69:211-4). In view of the identity of HSRTLIPE and the hedg7 3'-flanking 15 region, together with their virtually indistinguishable chromosomal localizations, we expect that the genes for hedg7 and the LIPE gene must be closely linked.

Nucleotide sequences encoding hedg-7 may be used to produce a purified oligo - or polypeptide using well known methods of recombinant DNA technology. Goeddel (1990, 20 Gene Expression Technology, Methods and Enzymology, Vol. 185, Academic Press, San Diego CA) is one among many publications which teach expression of an isolated nucleotide sequence. The oligopeptide may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which the nucleotide sequence was derived or from a different species. Advantages of producing an oligonucleotide by 25 recombinant DNA technology include obtaining adequate amounts of the protein for purification and the availability of simplified purification procedures.

Cells transformed with DNA encoding HEDG-7 may be cultured under conditions suitable for the expression of T7Gs, their extracellular, transmembrane or intracellular 30 domains and recovery of such peptides from cell culture. HEDG-7 (or any of its domains) produced by a recombinant cell may be secreted, expressend on cellular membranes or may be contained intracellularly, depending on the particular genetic construction used. In general, it is more convenient to prepare recombinant proteins in secreted form. Purification

steps vary with the production process and the particular protein produced. Often an oligopeptide can be produced from a chimeric nucleotide sequence. This is accomplished by ligating the nucleotides from hedg-7 or a desired portion of the polypeptide to a nucleic acid sequence encoding a polypeptide domain which will facilitate protein purification (Kroll DJ et al (1993) DNA Cell Biol. 12:441-53).

In addition to recombinant production, fragments of HEDG-7 may be produced by direct peptide synthesis using solid-phase techniques (e.g. Stewart et al (1969) Solid-Phase Peptide Synthesis, WH Freeman Co., San Francisco QA; Merrifield J (1963) J Am Chem. Soc. 85:2149-2154). Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Foster City, CA) in accordance with the instructions provided by the manufacturer. Additionally, a particular portion of HEDG-7 may be mutated during direct synthesis and combined with other parts of the peptide using chemical methods.

HEDG-7 for antibody induction does not require biological activity; however, the protein must be antigenic. Peptides used to induce specific antibodies may have an aa sequence consisting of at least five amino acids (aa), preferably at least 10 aa. They should mimic a portion of the aa sequence of the protein and may contain the entire aa sequence of a small naturally occurring molecule such as HEDG-7. An antigenic portion of HEDG-7 may be fused to another protein such as keyhole limpet hemocyanin, and the chimeric molecule used for antibody production.

Antibodies specific for HEDG-7 may be produced by inoculation of an appropriate animal with the polypeptide or an antigenic fragment. An antibody is specific for HEDG-7 if it is produced against an epitope of the polypeptide and binds to at least part of the natural or recombinant protein. Antibody production includes not only the stimulation of an immune response by injection into animals, but also analogous processes such as the production of synthetic antibodies, the screening of recombinant immunoglobulin libraries for specific-binding molecules (e.g. Orlandi R et al (1989) PNAS 86:3833-3837, or Huse WD et al (1989) Science 256:1275-1281) or the in vitro stimulation of lymphocyte populations. Current technology (Winter G and Mistein C (1991) Nature 349:293-299) provides for a number of highly specific binding reagents based on the principles of antibody formation. These techniques may be adapted to produce molecules which specifically bind HEDG-7s.

The intracellular carboxy-terminal domain is usually involved in interactions with intracellular proteins, including those that transduce intracellular signals upon receptor activation. The HEDG-7 intracellular domain is located at amino acids 305-384 as set out in 5 Figure 2A (SEQ ID NO:3). Furthermore, the carboxy-terminal domain is usually hydrophilic and highly antigenic relative to the receptor polypeptide as a whole. In addition, this domain normally shows greatly reduced conservation compared with other domains, and hence comprises a polypeptide sequence that is most unique to a given T7G receptor. Due to this diversity, this domain has special importance in the development of specific antibodies that 10 can be used in diagnosis of HEDG-7 related diseases, identification of HEDG-7 expressing cell populations within a tissue or cell type, or for purification and isolation of polypeptides containing this sequence. Since multiple epitopes are recognized by polyclonal antibodies, a polypeptide of this length may contain several distinct epitopes, or epitopes only created by close proximity of non-adjacent peptide sequences due to folding a tertiary structure of the 15 polypeptide.

An additional embodiment of the subject invention is the use of HEDG-7 specific antibodies, inhibitors, ligands or their analogs as bioactive agents to treat inflammation or disease including, but not limited to viral, bacterial or fungal infections; allergic responses; 20 mechanical injury associated with trauma; hereditary diseases; lymphoma or carcinoma; or other conditions which activate the genes of kidney, lung, heart, lymphoid or tissues of the nervous system.

Bioactive compositions comprising agonists, antagonists, receptors or inhibitors of 25 HEDG-7 may be administered in a suitable therapeutic dose determined by any of several methodologies including clinical studies on mammalian species to determine maximal tolerable dose and on normal human subjects to determine safe dose. Additionally, the bioactive agent may be complexed with a variety of well established compounds or compositions which enhance stability or pharmacological properties such as half-life. It is 30 contemplated that the therapeutic, bioactive composition may be delivered by intravenous infusion into the bloodstream or any other effective means which could be used for treating problems involving aberrant expression of the EDG-7 gene.

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

## 5 EXAMPLES

### Example 1: Amplification of partial mouse edg-7 DNA by polymerase chain reaction (PCR)

The Genbank database was surveyed for expressed sequence tags (EST's) 10 representing novel G protein-coupled receptors (GPCRs) related to edg-1. This was done by searching the EST subset of Genbank via the Internet using the TBLASTN algorithm. This tool allows a given amino acid sequence (in this case the human edg-1 polypeptide) to be compared to each partial cDNA in the EST database, first translated into all 6 possible reading frames. The sequences showing the most relatedness to the edg-1 polypeptide were 15 then classified as representing known (90% identical or more) or unknown (<90% identical) genes.

This analysis revealed 1 EST (Genbank accession number AA451451, derived from mouse mammary gland cDNA), which appeared to represent a new edg-related gene. This 20 gene is referred to herein as mouse edg-7 or medg. This EST was used to further search the EST database for overlapping EST's derived from the same gene. This search revealed 1 additional EST (Genbank accession number AA254425, derived from mouse lymph node), which shared 100% identity over a 104 bp overlap. These 2 EST's were aligned and used to build an extended mouse edg-7 sequence. The partial translation product (213 aa) of this 25 sequence showed 45.0% identity and 51.7% similarity to human edg-3, 42.8% identity and 54.6% similarity to human edg-1, and 32.9% identity and 42.8% similarity to rat edg-2. Thus, edg-7 appears to reside within the edg family of GPCRs, perhaps within the edg-1 subfamily (together with edg-3 and edg-4) rather than the edg-2 subfamily (with edg-5 and edg-6).

30 The assembled mouse partial edg-7 sequence was used to design oligonucleotide primers for PCR amplification. Since the normal tissue distribution of edg-7 was unknown, genomic DNA was used as the template for PCR. If introns interrupt the coding sequence of edg-7, such a PCR reaction might not be expected to work. However, our analysis of the

highly-related genes edg-1 and edg-3 showed that no introns occur within the open reading frame. This differs from the slightly more distant edg-2 grouping of edg genes, in which 1 or more introns interrupt the open reading frame.

5                  Mouse genomic DNA was amplified using the Expand™ PCR kit from Boehringer-Mannheim (Cat. 1681-842) in the following reaction:

10                6 µl    10X PCR Buffer 1 (Expand™ kit)  
                  8.4 µl 2.5 mM mixture of each dNTP  
                  1.8 µl 10 µM medg7-F2 primer: [5'-TATGTGCTCTTTGTGTGGTGGTC-3']  
                  1.8 µl 10 µM medg7-R1 primer: [5'-AAGGTTCTTGTGTCCTGTCCCTTC-3']  
                  0.9 µl Expand™ polymerase enzyme (3 units)  
                  40.1 µl water  
                  1 µl    mouse genomic DNA (Promega; Cat. G309A)

15                PCR conditions:  
Incubate:      94 °C for 2 min  
30 cycles:     92 °C for 1 min  
                  55 °C for 2 min  
20                68 °C for 1 min  
Incubate:      68 °C for 8 min  
Hold:           4 °C

25                The amplified 600 bp DNA fragment (designated as sample no. 806-33) was purified using QIAquick PCR purification kit from Qiagen (Cat. 28106) and directly sequenced with an ABI 377 automated sequencer in-house, using the PCR primers to prime the sequencing reactions, and fluorescent dideoxy- terminator nucleotides to determine the sequence as per the manufacturer's suggested protocol.

30                Example 2: Isolation of human genomic DNA clones containing the edg-7 gene

We made use of a commercially available human genomic DNA library, arrayed at high density on filter membranes, and probed these by hybridization with the radiolabeled

partial mouse edg-7 cDNA of Example 1. Each arrayed clone contains a genomic DNA insert of about 120 kb in the BAC (bacterial artificial chromosome) plasmidic vector.

1. Screening of a human genomic DNA library consisting of high-density arrayed BAC clones with a radiolabeled degenerate oligonucleotide:

A degenerate edg-7 oligonucleotide Edg7-1

[5'-CTGCTCYASCMTSCTGCCCTCTACTCCAAG-3'] was used to screen filters containing the high density arrayed BAC library (Genome Systems Inc.; Cat. BAC-5231) by hybridization under the following conditions:

Hybridize at 50°C overnight in:

5X SSPE (2X SSPE is 0.36 M NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 20 mM EDTA, pH 7.4)

5X Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA)

15 25 µg/ml herring sperm DNA

Wash 2 times for 30 min each at room temperature in:

2X SSPE

1% SDS

Wash 2 times for 20 min each at 50°C in:

20 2X SSPE

1% SDS

Wash 2 times for 20 min each at 50°C in:

1X SSPE

0.5% SDS

25

After rescreening these clones with radiolabeled partial mouse edg-7 PCR probe (sample 806-33, described above) one positive clone, 460D20 (identified by GSI as control no. 18241), was identified.

- 30 2. Screening of a human genomic DNA library consisting of high-density arrayed PAC clones with a radiolabeled partial mouse DNA (sample 806-33):

Random-primed, radiolabeled mouse edg-7 DNA sample 806-33 was used to screen filters containing the high density arrayed PAC library (Genome Systems Inc.; Cat. PAC-6541) by hybridization under the following conditions:

Hybridize at 60 °C overnight in:

5      5X SSPE (2X SSPE is 0.36 M NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 20 mM EDTA, pH 7.4)

      5X Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA)

      25 µg/ml herring sperm DNA

Wash 2 times for 30 min each at room temperature in:

10     2X SSPE  
      0.1% SDS

Wash 2 times for 20 min each at 50 °C in:

      2X SSPE  
      0.1% SDS

15     Wash 2 times for 20 min each at 50 °C in:  
      0.2X SSPE  
      0.1% SDS

One positive PAC clone, 230F18 (identified by GSI as control no. 19520) was identified and  
20 ordered from Genome Systems Inc. Rescreening of this clone with radiolabeled probe  
verified that the clone ordered was the one that gave the positive signal on hybridization  
screening.

Example 3: Subcloning of full-length human edg-7 DNA

25     Southern blot analysis of the BAC and PAC clones using radiolabeled mouse edg-7  
DNA sample 806-33 revealed the following hybridizing restriction fragments: HinDIII: >10  
kb; EcoRI: >10 kb; MscI: 1.5 kb; DdeI: 0.7 kb. Each BAC or PAC clone was shotgun-  
subcloned into pBluescript II (SK+) (Stratagene; Cat. 212205) and the resulting colonies were  
30 screened by hybridization to radiolabeled mouse edg-7 DNA 806-33.

Clones containing the 1.5 kb MscI and 0.7 kb DdeI DNA fragments were sequenced.  
Sequence analysis confirmed that these clones contained portions of the human edg-7 gene.

Based on the partial human edg-7 sequence, new primers were synthesized and more human edg-7 sequence was obtained from pBluescript clones containing the >10 kb HinDIII and EcoRI fragments. Additional sequencing primers were designed, and in this fashion the complete coding sequence of human edg-7 was determined. Furthermore, like other edg-1 subfamily members, no introns were found within the coding region of the edg-7 gene.

Two new primers were designed to facilitate the subcloning of an edg-7 DNA fragment encoding a full-length expressible edg-7 polypeptide into a eukaryotic expression vector. These were used to amplify human genomic DNA in a PCR reaction carried out under the following conditions:

4 µl 10X PCR Buffer 3 (Expand™ kit)  
0.8 µl 2.5 mM mixture of each dNTP  
1.2 µl 10 µM H7-F14 primer: [5'-GGAGGCCATGAACGCCACGGGGAC-3']  
15 1.2 µl 10 µM H7-R19 primer: [5'-AACTTCAGATGCTCCGCACGCTGGAG-3']  
0.6 µl Expand™ polymerase enzyme (2 units)  
31.2 µl water  
1 µl human genomic DNA (Progmege; Cat. G304A)

20 PCR conditions:

Incubate : 94 °C for 2 min  
30 cycles: 94 °C for 1 min  
              60 °C for 30 sec  
              68 °C for 1.5 min  
25 Incubate: 68 °C for 8 min  
Hold:      4 °C

The amplified 1.25 kb DNA fragment (designated as sample no. 1123-6) was purified using QIAquick PCR purification kit. This DNA was next re-amplified with edg-7 primers modified to contain restriction sites for convenient sub-cloning into the eukaryotic expression vector pcDNA3 (Invitrogen; discontinued), under the following conditions:

10 µl 10X PCR Buffer 3 (Expand™ kit)

2.0  $\mu$ l 2.5 mM mixture of each dNTP

3.0  $\mu$ l 10  $\mu$ M H7-F24: [5'-

TTTAAAAAGCTTGGAGGCCATAACGCACGGGGAC-3']

3.0  $\mu$ l 10  $\mu$ M H7-R21: [5'-

5 TATATATCTAGAACTTCAGATGCTCCGACGCTGGAG-3']

1.5  $\mu$ l Expand<sup>TM</sup> polymerase enzyme (5 units)

79.5  $\mu$ l water

1  $\mu$ l human edg-7 PCR DNA sample 1123-6

10 PCR conditions:

Incubate : 94 °C for 2 min

30 cycles: 92 °C for 1 min

55 °C for 30 sec

68 °C for 1.5 min

15 Incubate: 68 °C for 8 min

Hold: 4 °C

The amplified DNA fragment (designated as sample no. 1125-4) was purified, restricted with HinDIII and XbaI, then cloned into the appropriately prepared pcDNA3 vector. A clone, pC3-20 hEdg7, containing the edg-7 insert was isolated and a large-scale plasmid preparation was prepared for DNA sequencing and for transfection and subsequent expression analysis in eukaryotic cells. Figure 1A illustrates the nucleotide sequence (SEQ ID NO:1) for hedg-7 derived the Bac and Pac clones. Figure 2A illustrates the TM regions and the predicted amino acid sequence (SEQ ID NO:3). Figure 3A is an alignment of the nucleotide sequence 25 with the amino acid sequence.

Example 4: Isolation of BAC clones containing the mouse edg-7 gene

The partial mouse edg-7 DNA sample 806-33 was used to screen filters containing 30 high-density arrayed mouse genomic DNA BAC clones from Genome Systems Inc. (Cat. BAC-4921) by filter hybridization. The following conditions were employed:

Hybridize at 64 °C overnight in:

5X SSPE

5X Denhardt's solution

25 µg/ml herring sperm DNA

Wash 2 times for 30 min each at room temperature in:

5      2X SSPE

0.1% SDS

Wash 2 times for 20 min each at 50°C in:

2X SSPE

0.1% SDS

10     Wash 2 times for 20 min each at 50°C in:

0.2X SSPE

0.1% SDS

Three positive clones were identified from this screen: 76N3 (GSI control no. 19983),

15     61L2 (control no. 19984) and 61O13 (control no. 19985). PCR amplification of the three clones with primers medg7-F2 and medg7-R1 (for details see Example 1) produced a 600 bp PCR product in each case, indicating that all 3 clones contain mouse edg-7 sequence. Considering the size of the inserts, it is reasonable to assume that at least 1 of the 3 clones may contain the complete mouse edg-7 gene.

20

Example 5: Cloning of partial rat edg-7 cDNA sequence

A. PCR amplification of a rat edg-7 cDNA fragment.

25     The primers medg7-F2 and medg7-R1 were used to amplify a rat hypothalamus cDNA library (RHT) prepared in-house. This library was synthesized from random-primed first strand cDNA and cloned unidirectionally into the HinDIII/NotI sites of the pcDNA3 expression vector. PCR was performed under the following conditions:

30     2 µl    10X PCR Buffer 1 (Expand™ kit)

2.8 µl 2.5 mM mixture of each dNTP

0.6 µl 10 µM medg7-F2 primer

0.6 µl 10 µM medg7-R1 primer

0.3  $\mu$ l Expand<sup>TM</sup> polymerase enzyme (1 unit)  
12.7  $\mu$ l water  
1  $\mu$ l RHT cDNA complete library miniprep DNA

5 PCR conditions:

Incubate : 92 °C for 2 min  
30 cycles: 92 °C for 40 sec  
              52 °C for 1 min  
              68 °C for 1 min  
10 Incubate: 68 °C for 8 min  
Hold:        4 °C

A 600 bp product was seen, demonstrating that the RHT cDNA library contained clone(s) representing the rat edg-7 gene.

15

B. Screening 2777-clone pools of RHT cDNA library for edg-7 clones.

A series of pools containing a calculated 2777 clones per pool were screened using the medg7-F2 and medg7-R1 primers under the conditions specified in A above. Out of 884  
20 pools screened, only 1 pool was positive for the 600 bp PCR product of the edg-7 PCR. This pool (no. 198) was used for further study.

C. Amplification of rat edg-7 cDNA from RHT pool 198.

25 By using 1 specific primer (medg7-F2 or medg7-R1) vs one vector-based primer, the cDNA insert from RHT pool 198 was amplified in 2 overlapping pieces. Since the cDNA inserts were directionally cloned, the appropriate combination of specific vs vector primer could easily be chosen. Reaction conditions are shown below:

30 Vector-based primers:

830F: [5'-TAGAGAACCCACTGCTTAC-3']

1186R: [5'-CCCAGAATAGAATGACACC-3']

2 µl 10X PCR Buffer 1 (Expand™ kit)  
2.8 µl 2.5 mM mixture of each dNTP  
0.6 µl 10 µM mouse edg-7 specific primer  
0.6 µl 10 µM vector primer  
5 0.3 µl Expand™ polymerase enzyme (2 units)  
12.7 µl water  
1 µl RHT pool 198 miniprep DNA

PCR conditions:

10 Incubate : 92 °C for 2 min  
30 cycles: 92 °C for 40 sec  
50 °C for 1 min  
68 °C for 3 min  
Incubate: 68 °C for 8 min  
15 Hold: 4 °C

The most prominent bands (800 bp from primer set 830F vs medg7-R1 and 1.3 kb from primer set medg7-F2 vs 1186R) were reamplified, purified and sequenced. The nucleotide sequence is presented in Figure 5 and the amino acid sequence is in Figure 6.

20 Example 6: Cloning and expression of HEDG7 from human Jurkat T-cell cDNA  
To determine the agonist specificity and demonstrate methods for using HEDG7 in drug discovery, it was desirable to isolate a full-length cDNA clone from a human source. For 25 this purpose, numerous cDNA libraries and first strand cDNA pools, were surveyed by PCR to identify sources for further analysis. From this survey, a commercially available cDNA library (Origene Technologies, Cat. DLH-115) prepared from the human Jurkat T-cell lymphoma cell line was chosen. The previously described PCR primers H7-F14 and H7-R19 were used as follows to amplify any full-length cDNA that might be found in this cDNA 30 library:

All PCR amplifications were carried out with the Expand™ PCR kit (Boehringer-Mannheim, Cat. 1681-842). The reaction contained the following reagents:

2  $\mu$ l 10x PCR Buffer 3  
0.4  $\mu$ l 25mM dNTP mix  
0.6  $\mu$ l Primer H7-F14 (10pmol/ $\mu$ l)  
5 0.6  $\mu$ l Primer H7-R19 (10pmol/ $\mu$ l)  
0.3  $\mu$ l Expand polymerase (3 units)  
15.1  $\mu$ l water  
1  $\mu$ l cDNA from Origene library DLH-115 (as supplied by manufacturer)

## 10 PCR conditions:

Incubate: 94°C for 2 min  
30 cycles: 94°C for 1 min  
60°C for 1 min  
68°C for 2 min  
15 Incubate: 68°C for 8 min  
Hold: 4°C

The PCR reaction (tracking number 80629-50) amplified a 1200 bp DNA fragment. This was used as template to re-amplify human edg7 with primers H7-F23 and H7-R21, described previously, containing restriction sites for subcloning into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Cat V790-20).

Each reaction contained the following reagents:

25 5  $\mu$ l 10x PCR Buffer 3  
1.0  $\mu$ l 25mM dNTP mix  
1.5  $\mu$ l Primer H7-F23 (10pm/ $\mu$ l)  
1.5  $\mu$ l Primer H7-R21 (10pm/ $\mu$ l)  
0.75  $\mu$ l Expand™ polymerase (5 units)  
30 39.25  $\mu$ l water  
1  $\mu$ l 80629-50 DNA

## PCR conditions:

Incubate: 94°C for 2 min

10 cycles: 94°C for 1 min

55°C for 1 min

5 68°C for 2 min

15 cycles: 94°C for 1 min

68°C for 3 min

Incubate: 68°C for 8 min

Hold: 4°C

10

The amplified fragments from PCR reactions 80630-21 and 80630-22 were purified using QIAquick PCR purification kit (Qiagen, Cat.28106), pooled and designated as insert hedg7-M. The hedg7-M cDNA was restricted with Hind III and Xba I, purified using the QIAquick PCR purification kit, then isolated after agarose gel electrophoresis and purified with the QIAquick Gel Extraction kit (Qiagen, Cat. 28704). The cDNA fragment was then subcloned into HindIII and XbaI restricted pcDNA3.1 (Invitrogen).

15

Miniprep DNA from 4 clones was tested by cotransfection into 293-EBNA cells (Invitrogen, Cat. R620-07) with a 2XSRE-Luciferase reporter gene constructed in-house. We previously determined that these cells are an ideal expression host for edg receptors that respond to sphingolipids, since only a single edg gene is expressed (the LPA receptor subtype edg-5, see U.S. S. N. 08/997,803), under normal culture conditions. All other cell types tested showed a complex edg receptor expression pattern, together with unacceptable responsiveness to S1P, SPC, LPA, or more than one of these lysolipids. Thus, the low endogenous expression of previously identified S1P receptor subtypes (edg-1, edg-3, edg-4/H218) and hedg7 (determined here) permits overexpression and functional analysis of these receptors in a relatively unresponsive cell background. From this preliminary cotransfection experiment, the expression clone pC3-hEdg7#M10 was chosen for further analysis. The nucleotide sequence (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:3) of the hedg-7 sequence from the pC3-hEdg7#M10 clone are shown in Figure 1B and Figure 2B, respectively. Furthermore, an alignment of the hedg-7 amino acid sequence from the pC3-hEdg7#M10 clone and from the genomic DNA sequence is shown in Figure 3B.

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The EBNA-293 cells when transfected with DNAs of clone pc3-hedg7#M10 and 2XSRE-Luciferase reporter gave a 5.3- and 6.5-fold response to 10  $\mu$ M S1P treatment in 2 independent experiments. (Figure 7)

5 Transient transfection protocol for 293-EBNA:

Day 1.

1) 100 mm plates of 293-EBNA with a confluence of ~80% were used for transfection.

2) SRE Reporter Gene Cotransfection: Expression plasmid (3.5 g) and reporter plasmid

10 (2xSREtk-p4Luc-zeo; 0.5  $\mu$ g) DNA samples were combined and diluted in 750  $\mu$ l of DMEM/F12 (serum-free media) and 20  $\mu$ l Plus Reagent (Lipofectamine Plus Kit, Life Technologies Cat. 10964-013), and incubated at room temperature for 15 min.

3) 30  $\mu$ l Lipofectamine Reagent (Lipofectamine Plus Kit) was diluted in 750  $\mu$ l DMEM/F12.

The diluted Lipofectamine was then combined with the DNA/Plus mixture and incubated at

15 RT for 15 min.

4) The 293-EBNA plates were washed once with PBS, and 5 ml DMEM/F12 was added to each plate.

5) DNA/Plus/Lipofectamine mixture was added to each plate of 293-EBNA cells. The plates were left for 3 hr at 37°C in a 5% CO<sub>2</sub> incubator.

20 6) The transfection medium was replaced with DMEM/F12 containing 10% FBS to recover overnight.

Day 2.

2) Transfected cells were harvested by trypsinization and 20,000 cells per well were plated in 25 96-well Blackview plates coated with poly D-lysine (Becton Dickinson Labware, Cat. 40640). Medium was DMEM/F12 plus 0.15% FBS. No cells were plated in the outside wells of the 96-well plate. Cells were returned to the incubator for 48 hr.

Day 4.

30 1) Media was removed and cells treated with compounds diluted in serum-free DMEM/F12 media and the following treatments: a) Untreated: Serum-free DMEM/F12; b) 10  $\mu$ M S1P in DMEM/F12 medium.

2) The cells were treated 5 hr in the 37°C incubator.

- 3) Luclite kit (Packard; Cat. 601691.1) was used for luciferase assay. All reagents were brought to room temperature before use.
- 4) Media was removed from each well. 50 µl 0.5M HEPES pH 7.8, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> was added to all wells of 96-well plate.
- 5) 5) Luclite substrate was made up and 50 µl substrate was added to each well as specified in the kit.
- 6) Plates were incubated at room temperature for 30 min.
- 7) After incubation, plates were counted in a 12-detector Packard Top Count on a program without dark delay.

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Example 7: Agonist Specificity of HEDG7

To determine the agonist specificity of HEDG-7, 293-EBNA cells were transfected with pc3-hedg7#M10, serum-deprived as described above, and treated in serum-free medium with 5 µM concentrations of SPC, S1P, LPA, lysophosphatiylcholine (LPC), edelfosine, psychosine, anandamide or 2-arachidonylglycerol. Control cells were treated with serum-free medium alone, and the SRE response was expressed as fold induction relative to this control. After 5 hr of treatment, luciferase activity was measured.

- 20 Results: Both SPC and S1P robustly induced expression of the SRE reporter gene in cells transfected with pc3-hedg7#M10 (Figure 8). In contrast, LPA, LPC and edelfosine failed to activate the SRE response, supporting the assignment of edg-7 as a S1P receptor subtype, along with edg-1, edg-3 and edg-4/H218. This finding is also in accord with the lack of coding region introns found in these 4 receptor genes.

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Example 8: Determination of relative potency and efficacy of HEDG7 receptor agonists

One aspect of the present invention is a method for using recombinant HEDG7 receptors in drug screening programs. Although the use of T7G receptors in high-throughput screening is well-known, no such screen has been reported for the HEDG-7 receptor. More specifically, the novel HEDG7 receptor presented herein can be used to identify and rank the relative potency and efficacy of potential agonists. These compounds may be useful inasmuch as they would be expected to modulate cellular or physiological responses to HEDG7

agonists, or to initiate or supplement HEDG7 signaling in cells where the receptor occurs. Equally, once a quantitative and reliable assay is established, it can readily be applied to identify and rank the relative potency and efficacy of receptor antagonists.

5 Transfection of a HEDG7 expression vector pc3-hedg7#M10 was carried out using Lipofectamine Plus (Life Technologies, Cat. 10964-013) according to manufacturer's instructions. The next day, transfected cells were harvested by trypsinization and replated at 30,000 cells per well in poly-(D)lysine-coated 96-well plates in medium containing 0.15% FBS. The next day, cells were treated in serum-free medium containing different 10 concentrations of various sphingolipids. To demonstrate the utility of HEDG7 in drug discovery, we tested the ligand specificity and responsiveness of HEDG7 in a similar manner.

15 Various concentrations of S1P, SPC, psychosine, glucospsychosine or dihydrosphingosine 1-phosphate (dihydro-S1P) were applied in triplicate to cells in 96-well plates, and luciferase levels were measured after 6 hr treatment. Results were tabulated in Microsoft Excel, and analyzed with GraphPad Prism software. EC<sub>50</sub> values were determined using a fixed Hill-slope equation, unless variable slope significantly improved the fit to the data. The luciferase response was expressed as fold response, after subtracting any 20 endogenous response in pcDNA3-transfected cells at a given concentration of compound. The experiment was repeated three times with similar results, and a representative experiment is shown in Figure 9.

25 Results: Table 1 summarizes the relative potency and efficacy of the compounds tested. The concentration-dependent response to these sphingolipids is shown in Figure 9.

Table 1. Potency and relative efficacy of sphingolipids in 293-EBNA cells transfected with pc3-hedg7#M10.

Compound	EC <sub>50</sub> (M)	Rank	Max. Fold	E <sub>Max</sub> (Percent)	Rank
S1P	1.87	1	3.59	100	1
SPC	3.95	2	3.59	100	1
Psychosine	N.A.	-	1.00	0	-
Glucospsychosine	N.A.	-	1.00	0	-

N.A. Since no response was seen at nontoxic concentrations, EC<sub>50</sub> and rank could not be determined.

From the results obtained here, it can be concluded that HEDG7 responds to both S1P and SPC as full agonists, though S1P showed a lower EC<sub>50</sub> than SPC. In contrast, psychosine and glucopsychosine both failed to activate HEDG7 at nontoxic concentrations. Published literature supports the existence of multiple receptors for S1P, the identity of at least some of these with SPC receptors, subtype-selective differences in the relative potencies of S1P and SPC, and the possible existence of receptor(s) for psychosine and glucopsychosine. Here we have demonstrated that HEDG7 is a receptor for S1P and SPC, but not psychosine or glucopsychosine. With a method for screening, S1P receptor subtypes and ranking relative potency and efficacy of analogs and/or organic heterocycles, there is little doubt that rapid improvements can be made on the medicinal chemistry of S1P. These novel compounds, in turn, can be used to treat hyper- or hypo-proliferative diseases, and modulate inflammatory and antigen-specific immune responses as described elsewhere in this document.

Example 9: Detection of hedg polynucleotides by hybridization with hedg

Hedg polynucleotides can vary through the introduction of natural or artificial mutations or through cloning and subsequent manipulations. Moreover, the mammalian homolog of a given gene usually varies by 10-30% from species to species, as a result of nucleotide changes that have accumulated through their divergent evolutionary history. Therefore, a method is provided herein for the detection and identification of hedg variants and other highly related genes.

The HEDG7 coding region of hedg is prepared by restriction of either pC3-hEdg7 or pc3-hedg7#M10 with HinDIII and XbaI, followed by cDNA insert purification using standard techniques after agarose gel electrophoresis. The cDNA insert may be labeled using <sup>32</sup>P-nucleotide end-labeling or random priming (several kits are commercially available), or through incorporation of non-natural nucleotides for later detection with antibodies by methods well known in the art. Nylon filters (e.g. Hybond N+, Cat. RPN132B) bearing a polynucleotide or mixture of polynucleotides are prepared by standard techniques. Examples

include Southern blots, filter lifts from bacterial colonies or bacteriophage plaques and the like.

The dried filters are rehydrated in water, then prehybridized in a sealable bag with 10  
5 ml (or enough to cover filters and seal the bag) of hybridization solution (48% deionized formamide, 4.8× SSC [20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0], 1× Denhardt's solution [50× Denhardt's is 1% Ficoll 400, 1% polyvinylpyrrolidone, 1% BSA (Pentax Fraction V)], 10% dextran sulfate, 0.1% sodium dodecyl sulfate [SDS]) for 1 hr or more at 42°C.

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Radiolabeled probe is added to 1 ml of sonicated herring sperm DNA (2 mg/ml) in a screw-cap tube and incubated in a boiling water bath for 10 min. Transfer the tube to ice, add 2 ml of hybridization solution and inject the probe solution into the sealed bag. Sufficient probe should be added to give 1 to 15 ng of radiolabeled probe/ml hybridization buffer (final volume) at  $>5 \times 10^7$  cpm/g DNA. Reseal the bag, mix thoroughly and incubate overnight at 42°C in a shaking or rotating water bath or incubator.

15 Wash filters three times with 500 ml of low-stringency wash buffer (2× SSC, 0.1% SDS) at RT for 15 min per wash, on a slowly rotating platform. Then wash two times with medium-stringency wash buffer (1× SSC, 0.1% SDS) at 65°C 15 min per wash. Dry the filters and expose to Phosphorimager cassette or autoradiography film. Positive spots or DNA bands are identified after subtraction of background or appropriate negative control samples (see below).

25

If needed, a DNA spot containing 10 pmol of the full-length hedg insert of pC3-hEdg7 can be used as a positive control (Pos) on the filter, and a DNA spot containing 10 pmol of full-length human edg-1 insert (edg-1 open reading frame only) can be used as a negative control (Neg). The full-length open reading frame of a test DNA (also 10 pmol) will be scored as a positive if the integrated optical density (IOD) of the radioactive probe hybridizing to the test DNA (Test) is greater than  $IOD_{Neg} + (IOD_{Pos} - IOD_{Neg})/2$ . Otherwise, the test DNA will be scored as negative. A positive test correlates with approximately at least 70 % identity, and more preferably at least 80-85 sequence identity. If a partial-length open

reading frame of the test gene is used, then the equivalent regions of edg-7 and edg-1 will be used as positive and negative controls, respectively, for hybridization.

Example 10: Antisense analysis

5           Knowledge of the correct, complete cDNA sequence of HEDG-7 enables its use as a tool for antisense technology in the investigation of gene function. Oligonucleotides, cDNA or genomic fragments comprising the antisense strand of hedg-7 are used either in vitro or in vivo to inhibit expression of the mRNA. Such technology is now well known in the art, and  
10          antisense molecules can be designed at various locations along the nucleotide sequences. By treatment of cells or whole test animals with such antisense sequences, the gene of interest is effectively turned off. Frequently, the function of the gene is ascertained by observing behavior at the intracellular, cellular, tissue or organismal level (e.g., lethality, loss of differentiated function, changes in morphology, etc.).

15           In addition to using sequences constructed to interrupt transcription of a particular open reading frame, modifications of gene expression is obtained by designing antisense sequences to intron regions, promoter/enhancer elements, or even to trans-acting regulatory genes. Similarly, inhibition is achieved using Hogeboom base-pairing methodology, also  
20          known as "triple helix" base pairing.

Example 11: Expression of HEDG-7

Expression of hedg-7 is accomplished by subcloning the cDNAs into appropriate  
25          expression vectors and transfecting the vectors into analogous expression hosts for example E.Coli. In a particular case, the vector is engineered such that it contains a promoter for  $\beta$ -galactosidase, upstream of the cloning site, followed by sequence containing the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase. Immediately following these eight residues is an engineered bacteriophage promoter useful for artificial priming and  
30          transcription and for providing a number of unique endonuclease restriction sites for cloning.

Induction of the isolated, transfected bacterial strain with IPTG using standard methods produces a fusion protein corresponding to the first seven residues of  $\beta$ -

galactosidase, about 15 residues of "linker", and the peptide encoded within the cDNA. Since cDNA clone inserts are generated by an essentially random process, there is one chance in three that the included cDNA will lie in the correct frame for proper translation. If the cDNA is not in the proper reading frame, it is obtained by deletion or insertion of the appropriate 5 number of bases using well known methods including in vitro mutagenesis, digestion with exonuclease III or mung bean nuclease, or the inclusion of an oligonucleotide linker of appropriate length.

The hedg-7 cDNA is shuttled into other vectors known to be useful for expression of 10 protein in specific hosts. Oligonucleotide primers containing cloning sites as well as a segment of DNA (about 25 bases) sufficient to hybridize to stretches at both ends of the target cDNA is synthesized chemically by standard methods. These primers are then used to amplify the desired gene segment by PCR. The resulting gene segment is digested with appropriate restriction enzymes under standard conditions and isolated by gel electrophoresis. 15 Alternately, similar gene segments are produced by digestion of the cDNA with appropriate restriction enzymes. Using appropriate primers, segments of coding sequence from more than one gene are ligated together and cloned in appropriate vectors. It is possible to optimize expression by construction of such chimeric sequences.

20 Suitable expression hosts for such chimeric molecules include, but are not limited to, mammalian cells such as Chinese Hamster Ovary (CHO) and human 293 cells, insect cells such as Sf9 cells, yeast cells such as *Saccharomyces cerevisiae*, and bacteria such as *E. coli*. For each of these cell systems, a useful expression vector also includes an origin of replication to allow propagation in bacteria and a selectable marker such as the  $\beta$ -lactamase 25 antibiotic resistance gene to allow plasmid selection in bacteria. In addition, the vector may include a second selectable marker such as the neomycin phosphotransferase gene to allow selection in transfected eukaryotic host cells. Vectors for use in eukaryotic expression hosts require RNA processing elements such as 3' polyadenylation sequences if such are not part of the cDNA of interest.

30

Additionally, the vector contains promoters or enhancers which increase gene expression. Such promoters are host specific and include MMTV, SV40, and metallothioneine promoters for CHO cells; trp, lac, tac and T7 promoters for bacterial hosts; and alpha factor,

alcohol oxidase and PGH promoters for yeast. Transcription enhancers, such as the rous sarcoma virus enhancer, are used in mammalian host cells. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced HEDG-7 are recovered from the conditioned medium and analyzed 5 using chromatographic methods known in the art. For example, HEDG-7 can be expressibly cloned into the expression vector pcDNA3, as exemplified herein. This product can be used to transform, for example, HEK293 or COS by methodology standard in the art. Specifically, for example, using Lipofectamine (Gibco BRL catalog no. 18324-020) mediated gene transfer.

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Example 12: Isolation of Recombinant HEDG-7

HEDG-7 is expressed as a chimeric protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains 15 include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen) between the purification domain 20 and the HEDG-7 sequence is useful to facilitate expression of HEDG-7.

Example 13: Testing of Chimeric T7Gs

Functional chimeric T7Gs are constructed by combining the extracellular and/or 25 transmembrane ligand-receptive sequences of a new isoform with the transmembrane and/or intracellular segments of a different T7G for test purposes. This concept was demonstrated by Kobilka et al (1988, Science 240:1310-1316) who created a series of chimeric  $\alpha$ 2- $\beta$ 2 adrenergic receptors (AR) by inserting progressively greater amounts of  $\alpha$ 2-AR 30 transmembrane sequence into  $\beta$ 2-AR. The binding activity of known agonists changed as the molecule shifted from having more  $\alpha$ 2 than  $\beta$ 2 conformation, and intermediate constructs demonstrated mixed specificity. The specificity for binding antagonists, however, correlated with the source of the domain VII. The importance of T7G domain VII for ligand recognition was also found in chimeras utilizing two yeast  $\alpha$ -factor receptors and is significant because

the yeast receptors are classified as miscellaneous receptors. Thus, functional role of specific domains appears to be preserved throughout the T7G family regardless of category.

In parallel fashion, internal segments or cytoplasmic domains from a particular isoform are exchanged with the analogous domains of a known T7G and used to identify the structural determinants responsible for coupling the receptors to trimeric G-proteins (Dohlman et al (1991) Annu Rev Biochem 60:653-88). A chimeric receptor in which domains V, VI, and the intracellular connecting loop from  $\beta$ 2-AR were substituted into  $\alpha$ 2-AR was shown to bind ligands with  $\alpha$ 2-AR specificity, but to stimulate adenylate cyclase in the manner of  $\beta$ 2-AR. This demonstrates that for adrenergic-type receptors, G-protein recognition is present in domains V and VI and their connecting loop. The opposite situation was predicted and observed for a chimera in which the V- > VI loop from  $\alpha$ 1-AR replaced the corresponding domain on  $\beta$ 2-AR and the resulting receptor bound ligands with  $\beta$ 2-AR specificity and activated G-protein-mediated phosphatidylinositol turnover in the  $\alpha$ 1-AR manner. Finally, chimeras constructed from muscarinic receptors also demonstrated that V- > VI loop is the major determinant for specificity of G-protein activity (Bolander FF, supra).

Chimeric or modified T7Gs containing substitutions in the extracellular and transmembrane regions have shown that these portions of the receptor determine ligand binding specificity. For example, two Ser residues conserved in domain V of all adrenergic and D catecholamine T7G receptors are necessary for potent agonist activity. These serines are believed to form hydrogen bonds with the catechol moiety of the agonists within the T7G binding site. Similarly, an Asp residue present in domain III of all T7Gs which bind biogenic amines is believed to form an ion pair with the ligand amine group in the T7G binding site.

Functional, cloned T7Gs are expressed in heterologous expression systems and their biological activity assessed (e.g. Marullo et al (1988) Proc Natl Acad Sci 85:7551-55; King et al (1990) Science 250:121-23). One heterologous system introduces genes for a mammalian T7G and a mammalian G-protein into yeast cells. The T7G is shown to have appropriate ligand specificity and affinity and trigger appropriate biological activation--growth arrest and morphological changes--of the yeast cells.

An alternate procedure for testing chimeric receptors is based on the procedure utilizing the P<sub>2u</sub> purinergic receptor (P<sub>2u</sub>) as published by Erb et al (1993, Proc Natl Acad Sci 90:104411-53). Function is easily tested in cultured K562 human leukemia cells because these cells lack P<sub>2u</sub> receptors. K562 cells are transfected with expression vectors containing either normal or chimeric P<sub>2u</sub> and loaded with fura-a, fluorescent probe for Ca<sup>++</sup>. Activation of properly assembled and functional P<sub>2u</sub> receptors with extracellular UTP or ATP mobilizes intracellular Ca<sup>++</sup> which reacts with fura-a and is measured spectrofluorometrically. As with the T7G receptors above, chimeric genes are created by combining sequences for extracellular receptive segments of any newly discovered T7G polypeptide with the nucleotides for the transmembrane and intracellular segments of the known P<sub>2u</sub> molecule. Bathing the transfected K562 cells in microwells containing appropriate ligands triggers binding and fluorescent activity defining effectors of the T7G molecule. Once ligand and function are established, the P<sub>2u</sub> system is useful for defining antagonists or inhibitors which block binding and prevent such fluorescent reactions.

15

Example 14: Production of HEDG-7 Specific Antibodies

Two approaches are utilized to raise antibodies to HEDG-7, and each approach is useful for generating either polyclonal or monoclonal antibodies. In one approach, denatured protein from reverse phase HPLC separation is obtained in quantities up to 75 mg. This denatured protein is used to immunize mice or rabbits using standard protocols; about 100 micrograms are adequate for immunization of a mouse, while up to 1 mg might be used to immunize a rabbit. For identifying mouse hybridomas, the denatured protein is radioiodinated and used to screen potential murine B-cell hybridomas for those which produce antibody. This procedure requires only small quantities of protein, such that 20 mg is sufficient for labeling and screening of several thousand clones.

In the second approach, the amino acid sequence of an appropriate HEDG-7 domain, as deduced from translation of the cDNA, is analyzed to determine regions of high antigenicity. Oligopeptides comprising appropriate hydrophilic regions, as illustrated in Figure 2A, are synthesized and used in suitable immunization protocols to raise antibodies. Analysis to select appropriate epitopes is described by Ausubel FM et al (supra). The optimal amino acid sequences for immunization are usually at the C-terminus, the N-terminus and

those intervening, hydrophilic regions of the polypeptide which are likely to be exposed to the external environment when the protein is in its natural conformation.

- Typically, selected peptides, about 15 residues in length, are synthesized using an
- 5 Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH; Sigma, St. Louis MO) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel FM et al, *supra*). If necessary, a cysteine is introduced at the N-terminus of the peptide to permit coupling to KLH. Rabbits are
- 10 immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity by binding the peptide to plastic, blocking with 1% bovine serum albumin, reacting with antisera, washing and reacting with labeled (radioactive or fluorescent), affinity purified, specific goat anti-rabbit IgG.

- Hybridomas are prepared and screened using standard techniques. Hybridomas of
- 15 interest are detected by screening with labeled HEDG-7 to identify those fusions producing the monoclonal antibody with the desired specificity. In a typical protocol, wells of plates (FAST; Becton-Dickinson, Palo Alto CA) are coated during incubation with affinity purified, specific rabbit anti-mouse (or suitable antispecies Ig) antibodies at 10 mg/ml. The coated wells are blocked with 1% BSA, washed and incubated with supernatants from hybridomas.
- 20 After washing the wells are incubated with labeled HEDG-7 at 1 mg/ml. Supernatants with specific antibodies bind more labeled HEDG-7 than is detectable in the background. Then clones producing specific antibodies are expanded and subjected to two cycles of cloning at limiting dilution. Cloned hybridomas are injected into pristane-treated mice to produce ascites, and monoclonal antibody is purified from mouse ascetic fluid by affinity
- 25 chromatography on Protein A. Monoclonal antibodies with affinities of at least  $10^8$  M<sup>-1</sup>, preferably  $10^9$  to  $10^{10}$  or stronger, are typically made by standard procedures as described in Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; and in Goding (1986) *Monoclonal Antibodies: Principles and Practice*, Academic Press, New York City, both incorporated herein by reference.

Example 15: Diagnostic Test Using HEDG-7 Specific Antibodies

Particular HEDG-7 antibodies are useful for investigating signal transduction and the diagnosis of infectious or hereditary conditions which are characterized by differences in the amount or distribution of HEDG-7 or downstream products of an active signaling cascade.

Diagnostic tests for HEDG-7 include methods utilizing antibody and a label to detect HEDG-7 in human body fluids, membranes, cells, tissues or extracts of such. The polypeptides and antibodies of the present invention are used with or without modification. Frequently, the polypeptides and antibodies are labeled by joining them, either covalently or noncovalently, with a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are known and have been reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, chromogenic agents, magnetic particles and the like. Patents teaching the use of such labels include US Patent No's. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced as shown in US Patent No.4,816,567, Incorporated herein by reference.

A variety of protocols for measuring soluble or membrane-bound HEDG-7, using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on HEDG-7 is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, DE et al (1983, J Exp. Med. 158:1211f).

Example 16: Purification of Native HEDG-7 Using Specific Antibodies

Native or recombinant HEDG-7 is purified by immunoaffinity chromatography using antibodies specific for HEDG-7. In general, an immunoaffinity column is constructed by covalently coupling the anti-TRH antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared from immune sera either by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia LKB Biotechnology, Piscataway NJ). Likewise, monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein 5 A. Partially purified immunoglobulin is covalently attached to a chromatographic resin such as CnBr-activated Sepharose (Pharmacia LKB Biotechnology). The antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturer's instructions.

Such immunoaffinity columns are utilized in the purification of HEDG-7 by preparing a fraction from cells containing HEDG-7 in a soluble form. This preparation is derived by solubilization of whole cells or of a subcellular fraction obtained via differential centrifugation (with or without addition of detergent) or by other methods well known in the art. Alternatively, soluble HEDG-7 containing a signal sequence is secreted in useful 15 quantity into the medium in which the cells are grown.

A soluble HEDG-7-containing preparation is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of HEDG-7 (e.g., high ionic strength buffers in the presence of detergent). Then, the column is eluted 20 under conditions that disrupt antibody/protein binding (e.g., a buffer of pH 2-3 or a high concentration of a chaotropic such as urea or thiocyanate ion), and HEDG-7 is collected.

#### Example 17: Drug Screening

This invention is particularly useful for screening therapeutic compounds by using HEDG-7 or binding fragments thereof in any of a variety of drug screening techniques. As HEDG-7 is a G protein coupled receptor any of the methods commonly used in the art may potentially be used to identify HEDG-7 ligands. For example, the activity of a G protein coupled receptor such as HEDG-7 can be measured using any of a variety of appropriate 25 functional assays in which activation of the receptor results in an observable change in the level of some second messenger system, such as adenylate cyclase, guanylyl cyclase, calcium mobilization, or inositol phospholipid hydrolysis. One such approach, measures the effect of ligand binding on the activation of intracellular second messenger pathways, using a reporter

gene. Typically, the reporter gene will have a promoter which is sensitive to the level of that second messenger controlling expression of an easily detectable gene product, for example, CAT or luciferase. Alternatively, the cell is loaded with a reporter substance, e.g., FURA, which detects alterations in the intracellular level of calcium, can be used to monitor 5 modulation of the receptor as a result of ligand binding. Thus, the present invention provides methods of screening for drugs or any other agents which affect signal transduction.

Alternatively, the polypeptide or fragment employed in such a test is either free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One 10 method of drug screening utilizes eukaryotic or prokaryotic host cells (or membrane preparations therefrom) which are stably transformed with recombinant nucleic acids expressing the polypeptide or fragment. Drugs are screened against such transformed cells in competition binding assays. Such cells, either in viable or fixed form, are used for standard binding assays.<sup>32</sup> P-labelled S1P could be used in such a competition binding assay for 15 HEDG-7. One measures, for example, the formation of complexes between HEDG-7 and the agent being tested. Alternatively, one examines the diminution in complex formation between HEDG-7 and a ligand (for example, S1P), caused by the agent being tested.

#### Example 18: Rational Drug Design

20 Herein, the goal of rational drug design is to produce structural analogs of biologically active phospholipids of interest or of small molecules with which they interact, agonists, antagonists, or inhibitors. Any of these examples are used to fashion drugs which are more active or stable forms of the phospholipid or which enhance or interfere with the function of a 25 phospholipid in vivo.

In one approach, the three-dimensional structure of a protein of interest, or of a protein-inhibitor complex, is determined by x-ray crystallography, by computer modeling or, most typically, by a combination of the two approaches. Both the shape and charges of the 30 polypeptide must be ascertained to elucidate the structure and to determine active site(s) of the molecule. Less often, useful information regarding the structure of a polypeptide is gained by modeling based on the structure of homologous proteins. In both cases, relevant structural information is used to design efficient inhibitors. Useful examples of rational drug

design includes molecules which have improved activity or stability as shown by Braxton S and Wells JA (1992, Biochemistry 31:7796-7801) or which act as inhibitors, agonists, or antagonists of native peptides as shown by Athauda SB et al (1993 J Biochem 113:742-46), incorporated herein by reference.

5

Example 19: Use and Administration of Antibodies, Inhibitors, or Antagonists

Antibodies, inhibitors, or antagonists of HEDG-7 (or other treatments to limit signal transduction, LST) provide different effects when administered therapeutically. LSTs are  
10 formulated in a nontoxic, inert, pharmaceutically acceptable aqueous carrier medium preferably at a pH of about 5 to 8, more preferably 6 to 8, although pH may vary according to the characteristics of the antibody, inhibitor, or antagonist being formulated and the condition to be treated. Characteristics of LSTs include solubility of the molecule, half-life and antigenicity/immunogenicity. These and other characteristics aid in defining an effective  
15 carrier.

LSTs are delivered by known routes of administration including but not limited to topical creams and gels; transmucosal spray and aerosol; transdermal patch and bandage; injectable, intravenous and lavage formulations; and orally administered liquids and pills  
20 particularly formulated to resist stomach acid and enzymes. The particular formulation, exact dosage, and route of administration is determined by the attending physician and varies according to each specific situation.

Such determinations are made by considering multiple variables such as the condition  
25 to be treated, the LST to be administered, and the pharmacokinetic profile of a particular LST. Additional factors which are taken into account include severity of the disease state, patient's age, weight, gender and diet, time and frequency of LST administration, possible combination with other drugs, reaction sensitivities, and tolerance/response to therapy. Long acting LST formulations might be administered every 3 to 4 days, every week, or once every  
30 two weeks depending on half-life and clearance rate of the particular LST.

Normal dosage amounts vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and

methods of delivery is provided in the literature; see US Patent Nos. 4,657,760; 5,206,344; or 5,225,212. Those skilled in the art employ different formulations for different LSTs.

Administration to cells such as nerve cells necessitates delivery in a manner different from that to other cells such as vascular endothelial cells.

5

It is contemplated that abnormal signal transduction, trauma, or diseases which trigger HEDG-7 activity are treatable with LSTs. These conditions or diseases are specifically diagnosed by the tests discussed above, and such testing should be performed in suspected cases of viral, bacterial or fungal infections: allergic responses; mechanical injury associated 10 with trauma; hereditary diseases; lymphoma or carcinoma; or other conditions which activate the genes of lymphoid or neuronal tissues.

#### Example 20: Production of Transgenic Animals

15 Animal model systems which elucidate the physiological and behavioral roles of the HEDG-7 receptor are produced by creating transgenic animals in which the activity of the HEDG-7 receptor is either increased or decreased, or the amino acid sequence of the expressed HEDG-7 receptor is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA 20 encoding a HEDG-7 receptor, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these HEDG-7 receptor sequences. The 25 technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native HEDG-7 receptors but does express, for example, an inserted mutant HEDG-7 receptor, which has replaced the native HEDG-7 receptor in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but 30 does not remove them, and so is useful for producing an animal which expresses its own and added HEDG-7 receptors, resulting in overexpression of the HEDG-7 receptors.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a HEDG-7 purified from a vector by methods well known in the art.

- 5 Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a piper puller)
- 10 and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse ( a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only
- 15 methods for inserting DNA into the egg cell, and is used here only for exemplary purposes.

Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific  
20 preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments.

**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. An isolated nucleotide sequence encoding a human EDG-7 receptor.
2. A biologically active fragment of the isolated nucleotide sequence of claim 1.
3. The biologically active fragment of claim 2 wherein said fragment is activated by S1P or LPA.
4. An isolated nucleotide sequence selected from the group consisting of:
  - (a) the nucleotide sequence comprising nucleotides 16- 1170 of SEQ ID NO:1;
  - (b) the nucleotide sequence comprising nucleotides 13-116 of SEQ ID NO:2;
  - (c) a nucleotide sequence with at least about 70% sequence identity to (a) or (b) and which hybridizes under stringent conditions to sequences (a) and (b), respectively;
  - (d) a nucleotide sequence which encodes the amino acid sequence of SEQ ID NO:3; and
  - (e) a nucleotide sequence which encodes the amino acid sequence of SEQ ID NO:4.
5. The isolated nucleotide sequence of Claim 4 wherein the nucleotide sequence is selected from the group consisting of:
  - (a) the nucleotide sequence of (a), (b), (d) or (e) of claim 4; and
  - (b) a nucleotide sequence with at least about 80-85% sequence identity to (a) or (b) of claim 4 and which hybridizes under stringent conditions to sequences (a) or (b) of claim 4.
6. The isolated nucleotide sequence of Claim 4 wherein the nucleotide sequence is selected from the group consisting of:
  - (a) the nucleotide sequence of (a), (b), (d) or (e) of claim 4; and
  - (b) a nucleotide sequence with at least about 95% sequence identity to (a) or (b) of claim 4 and which hybridizes under stringent conditions to sequences (a) or (b) of claim 4.
7. The isolated nucleotide sequence of Claim 6 wherein the nucleotide sequence encodes a HEDG-7 receptor which is activated by S1P or SPC.

8. The complement of the nucleotide sequence of Claim 6.
9. An expression vector comprising the nucleotide sequence of Claim 6.
10. A host cell comprising the expression vector of Claim 9.
11. The isolated and purified amino acid sequence for the HEDG-7 receptor encoded by the nucleotide sequence of claim 6 or a biological active portion thereof.
12. The isolated and purified amino acid sequence of claim 11 comprising the amino acid sequence of SEQ ID NO:3 or SEQ ID NO:4 or a biological active portion thereof.
13. The isolated nucleotide sequence of Claim 4 wherein the nucleotide sequence is the nucleotide sequence of (d) or (e) of Claim 4.
14. A hybridization probe incorporating at least 15 contiguous nucleotides of the nucleotide sequence of Claim 4.
15. A method of screening compounds to identify HEDG-7 ligands comprising the steps of:
  - (a) culturing cells which express the HEDG-7 receptor or with a membrane preparation obtained therefrom;
  - (b) incubating said compounds with said cells or said membrane preparation therefrom; and
  - (c) determining whether binding between the HEDG-7 receptor and the compound has occurred.
16. A HEDG-7 ligand identified by the method of claim 15.
17. A method of screening compounds to identify HEDG-7 antagonists comprising the steps of:
  - (a) culturing cells which express the HEDG-7 receptor or with a membrane preparation obtained therefrom;

- (b) contacting said cells with a mixture comprising an agonist and said compound to be tested for antagonist activity at said receptor; and
- (c) determining the degree of antagonist activity by measuring a response indicative of the degree of binding between said agonist and HEDG-7 and comparing this measured response with a standard response for binding between HEDG-7 and said agonist absent said compound.

18. An antagonist identified by the method of claim 17.

## FIGURE 1A

Human edg-7 sequence derived from BAC clone 460D20 (Genome Systems control no. 18241) and PAC clone 230F18 (control no. 19520). Translation starts at nt 16 and ends with a stop codon at nt 1168-1170. Translation initiation and termination codons are underlined below.

1   CCCCCGGGGG AGGCCATGAA CGCCACGGGG ACCCCGGTGG CCCCCGAGTC  
51   CTGCCAACAG CTGGCGGCCG GCGGGCACAG CGGGCTCATT GTTCTGCACT  
101   ACAACCACTC GGGCCGGCTG GCCGGGCGCG GGGGGCCGGA GGATGGCGGC  
151   CTGGGGGCC TGCGGGGCT GTCGGTGGCC GCCAGCTGCC TGGTGGTGCT  
201   GGAGAACTTG CTGGTGCTGG CGGCCATCAC CAGCCACATG CGGTCGCGAC  
251   GCTGGGTCTA CTATTGCCCTG GTGAACATCA CGCTGAGTGA CCTGCTCACG  
301   GGCGCGGCCT ACCTGGCAA CGTGCTGCTG TCGGGGGCC GCACCTTCCG  
351   TCTGGCGCCC GCCCAGTGGT TCCTACGGGA GGGCCTGCTC TTCACCGCCC  
401   TGGCCGCCCTC CACCTTCAGC CTGCTCTCA CTGCAGGGGA GCGCTTGCC  
451   ACCATGGTGC GGCCGGTGGC CGAGAGCGGG GCCACCAAGA CCAGCCGCGT  
501   CTACGGCTTC ATCGGCCTCT GCTGGTGCT GGCGCGCTG CTGGGGATGC  
551   TGCCTTGCT GGGCTGGAAC TGCCTGTGCG CCTTGACCG CTGCTCCAGC  
601   CTTCTGCCCTC TCTACTCCAA GCGCTACATC CTCTTCTGCC TGGTGATCTT  
651   CGCCGGCGTC CTGGCCACCA TCATGGGCCT CTATGGGGCC ATCTCCGCC  
701   TGGTGCAGGC CAGCGGGCAG AAGGCCCCAC GCCCAGCGGC CCGCCGCAAG  
751   GCCCGCCGCC TGCTGAAGAC GGTGCTGATG ATCCTGCTGG CCTTCCTGGT  
801   GTGCTGGGC CCACTCTTCG GGCTGCTGCT GGCGGACGTC TTTGGCTCCA  
851   ACCTCTGGC CCAGGAGTAC CTGCGGGCA TGGACTGGAT CCTGGCCCTG  
901   GCCGTCTCA ACTCGGCGGT CAACCCCATC ATCTACTCCT TCCGCAGCAG  
951   GGAGGTGTGC AGAGCCGTGC TCAGCTTCCT CTGCTGCCGG TGTCTCCGGC  
1001   TGGGCATGCG AGGGCCCGGG GACTGCCTGG CCCGGGCCGT CGAGGCTCAC  
1051   TCCGGAGCTT CCACCACCGA CAGCTCTCTG AGGCCAAGGG ACAGCTTTCG  
1101   CGGCTCCCGC TCGCTCAGCT TTGGATGCG GGAGCCCCCTG TCCAGCATCT  
1151   CCAGCGTGC GAGCATCTGA AGTTGCAGTC TTGCGTGTGG ATGGTGCAGC  
1201   CACCGGGTGC GTGCCAGGCA GGCCCTCCTG GGGTACAGGA AGCTGTGTGC  
1251   ACGCAGCCTC GCCTGTATGG GGAGCAGGGA ACGGGACAGG CCCCCATGGT  
1301   CTTCCCGGTG GCCTCTCGGG GCTCTGACG CCAAATGGC TTCCCATGGT  
1351   CACCCCTGGAC AAGGAGGCAA CCACCCACC TCCCCGTAGG AGCAGAGAGC  
1401   ACCCCTGGTGT GGGGGCGAGT GGGTCCCCA CAACCCCGCT TCTGTGTGAT  
1451   TCTGGGAAG TCCCGGCCCC TCTCTGGGCC TCAGTAGGGC TCCCAGGCTG  
1501   CAAGGGGTGG ACTGTGGGAT GCATGCCCTG GCAACATTGA AGTTCGATCA  
1551   TGGTACGTGA TGTTGCGGCC TCTTATTCCC TGGTGCAGTC ATGTGTGGGG  
1601   GCCGTGGCTC AGGGGGGCTG TGGATCTAGG GGCAGCCGGG TGTGTCTTTG  
1651   CTAGAGAGGG CCACGGGCCA GTGCCCTGTG AGGGTGGAGA GTGTGTGTGT  
1701   GTGTGTGTGT GTGTGTGTGG ACAACYCTCTG GGCAGTGGGG GAAGTGGGGG

## FIGURE 1A (cont.)

1751 TGACAATGAC AGTTAATGCC GCTCTTCTTG TTCACTTCCC CTTTAGAAAT  
1801 GGCAGGGCCC ATGCCCATC TCTGGCYTCT GCATCTTTG GGGACCCACT  
1851 CTCTGGGCT GGCAAGAGGCA CCACCTTGGC TTCCCTGGGCT GGGGGAATCT  
1901 TCCCTCACAT CCCCTTCAGC ATGAACGGCC TCGGCTTCC CGGTGGGTAA  
1951 AACAGTTAA TCACTGAAGC CGAACGACAG GGTTGATGGT ACACGCTCCC  
2001 CGCCAGCCAC AGGGGCTGAC GACTGCCTGC CCCGTGAAAC TCCAGTGGAG  
2051 ACGTTTCAGC TCCACACCATT CAAGTATGGG AGACGCCAGC CCCACGGGGC  
2101 TACGGTCAA GCAGATAACT GAATTTCGAA GTGTAGGTTG TGTTTAATTT  
2151 GAATCTGTTT ATATTCGGT AGCCCCATGG GGCGGGTGGG GGGGATCCAC  
2201 TAGTTCTAGA GCGGCCGCCA CCGCGGTGGA GCTCCAGYWT TWGWTCCCKT  
2251 TAGTGAGGGT TAATTGCGCG

**Figure 1B**

Sequence of the cDNA insert of clone pc3-hedg7#M10. Translation starts at nt 13 and ends with a stop codon at nt 1165-1167.

hedg7#M10.seq Length: 1176

1 AAGCTTGCAC CCATGAACGC CACGGGGACC CCGGTGGCCC CCGAGTCCTG  
51 CCAACAGCTG CGGGCCGGCG GGCACAGCCG GCTCATTGTT CTGCACTACA  
101 ACCACTCGGG CCCGCTGGCC GGGCGCGGGG GGCCGGAGGA TGGCGGCCCTG  
151 GGGGCCCTGC GGGGGCTGTC GGTGGCCGCC AGCTGCCTGG TGTTGCTGGA  
201 GAACTTGCTG GTGCTGGCGG CCATCACCAAG CCACATGCGG TCGCGACGCT  
251 GGGTCTACTA TTGCCTGGTG AACATCACGC TGAGTGACCT GCTCACGGC  
301 GCGGCCTACC TGGCCAACGT GCTGCTGTCG GGGGGCCCGCA CCTTCCGTCT  
351 GGCGCCCGCC CAGTGGTTCC TACGGGAGGG CCTGCTCTTC ACCGCCCTGG  
401 CCGCCTCCAC CTTCAGCCTG CTCTTCACTG TAGGGGAGCG CTTTGCCACC  
451 ATGGTGCGGC CGGTGGCCGA GAGCGGGGCC ACCAAGACCA GCCGCGTCTA  
501 CGGCTTCATC GGCTCTGCT GGCTGCTGGC CGCGCTGCTG GGGATGCTGC  
551 CTTTGCTGGG CTGGAACCTGC CTGTGCGCCT TTGACCGCTG CTCCAGCCTT  
601 CTGCCCTCT ACTCCAAGCG CTACATCCTC TTCTGCCTGG TGATCTTCGC  
651 CGGCGTCCCTG GCCACCATCA TGGGCCTCTA TGGGGCCATC TTCCGCCTGG  
701 TGCAGGCCAG CGGGCAGAAC GCCCCACGCC CAGCGGCCCG CCGCAAGGCC  
751 CGCCGCCTGC TGAAGACGGT GCTGATGATC CTGCTGGCCT TCCTGGTGTG  
801 CTGGGGCCCA CTCTCGGGC TGCTGCTGGC CGACGTCTTT GGCTCCAACC  
851 TCTGGGCCCA GGAGTACCTG CGGGGCATGG ACTGGATCCT GGCCCTGGCC  
901 GTCCTCAACT CGGCGGTCAA CCCCATCATC TACTCCTTCC GCAGCAGGG  
951 GGTGTGCAGA GCGGTGCTCA GCTTCCTCTG CTGCGGGTGT CTCCGGCTGG  
1001 GCATGCGAGG GCCCGGGGAC TGCCCTGGCCC GGGCCGTGGA GGCTCACTCC  
1051 GGAGCTTCCA CCACCGACAG CTCTCTGAGG CCAAGGGACA GCTTTGCGGG  
1101 CTCCCGCTCG CTCAGCTTTC GGATGCGGGA GCCCCGTGTC AGCAGCTCCA  
1151 GCGTGCAGGAG CATCTGAAGT TCTAGA

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**FIGURE 2A**

Features of the human edg-7 polypeptide.

**1. Seven-transmembrane topology typical of G protein-coupled receptors:**

N-ter extracellular domain: 1-49

TM-1: 50-70

IL-1: 71-81

TM-2: 82-105

EL-1: 106-124

TM-3: 125-143

IL-2: 144-163

TM-4: 164-182

EL-2: 183-199

TM-5: 200-224

IL-3: 225-250

TM-6: 251-272

EL-3: 273-285

TM-7: 286-304

C-ter intracellular domain: 305-384

**2. Potential N-glycosylation sites: N-2, N-30****3. Potential phosphorylation sites: S-77, T-159, S-308, S-360, S-380**

Predicted amino acid sequence of the HEDG7 polypeptide.

1 MNATGTPVAP ESCQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR  
51 GLSVAASCLV VLENLLVLAA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL  
101 ANVLLSGART FRLAPAQWFL REGLLLFTALA ASTFSLLFTA GERFATMVRP  
151 VAESGATKTS RVYGFIGLCW LLAALLGMMP LLGWNCLCAF DRCSSLLPLY  
201 SKRYILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL  
251 KTVLMIALLAF LVCWGPLFGL LLADVFGSNL WAQEYLRGMD WILALAVLNS  
301 AVNPPIIYSFR SREVCRAVLS FLCCGCLRIG MRGPGDCLAR AVEAHSGAST  
351 TDSSLRPRDS FRGSRSLSFR MREPLSSIIS VRSI

## Figure 2B

Amino acid sequence of HEDG7 from expression clone pc3-hedg7#M10. The polypeptide product of pc3-hedg7#M10 was deduced from the sequence of the cDNA insert, and this variant of HEDG7 was designated HEDG7#M10.

1 MNATGTPVAP ESCQQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR  
51 GLSVAASCLV VLENLLVLAA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL  
101 ANVILLSGART FRLAPAQWFL REGLLFTALA ASTFSLLFTV GERFATMVRP  
151 VAESGATKTS RVYGFIGLCW LLAALLGMLP LLGWNCLCAF DRCSSLLPLY  
201 SKRYIILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL  
251 KTVLMIILLAF LVCWGPLFGL LLADVFVGSNL WAQEYLRGMD WILALAVLNS  
301 AVNPIIYSFR SREVCRAVLS FLCCGCLRLG MRGPGDCLR AVEAHSGAST  
351 TDSSLRPRDS FRGSRSLSFR MREPLSSSSS VRSI.

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Figure 3A

Alignment of human edg-7 DNA sequence (clone pc3-hedg7#m10) with the encoded HEDG7 polypeptide.

```

13 ATGAACGCCACGGGGACCCCGGTGGCCCCCGAGTCCTGCCAACAGCTGGC 62
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
1 MetAsnAlaThrGlyThrProValAlaProGluSerCysGlnGlnLeuAl 17

63 GGCCGGCGGGCACAGCCGGCTCATTGTTCTGCACTACAACCACCTGGGCC 112
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
18 aAlaGlyGlyHisSerArgLeuIleValLeuHisTyrAsnHisSerGlyA 34

113 GGCTGGCCGGGCGCGGGGGCCGGAGGATGGCGGCCTGGGGGCCCTGCGG 162
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
35 rgLeuAlaGlyArgGlyGlyProGluAspGlyGlyLeuGlyAlaLeuArg 50

163 GGGCTGTCGGTGGCCGCCAGCTGCCTGGTGGTGGCTGGAGAACTTGCTGGT 212
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
51 GlyLeuSerValAlaAlaSerCysLeuValValLeuGluAsnLeuLeuVa 67

213 GCTGGCGGCCATCACAGCCACATGCGGTGCGACGCTGGGTCTACTATT 262
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
68 lLeuAlaAlaIleThrSerHisMetArgSerArgArgTrpValTyrTyrC 84

263 GCCTGGTGAACATCACGCTGAGTGACCTGCTCACGGCGCGCCCTACCTG 312
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
85 ysLeuValAsnIleThrLeuSerAspLeuLeuThrGlyAlaAlaTyrLeu 100

313 GCCAACGTGCTGCTGCGGGGCCGCACCTTCCGTCTGGCGCCCGCCCA 362
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
101 AlaAsnValLeuLeuSerGlyAlaArgThrPheArgLeuAlaProAlaGl 117

363 GTGGTTCCCTACGGGAGGGCCTGCTCTTCACCGCCCTGGCCGCCCTCACCT 412
|||||||||||||||||||||||||||||||||||||||||||||||||||||||
118 nTrpPheLeuArgGluGlyLeuLeuPheThrAlaLeuAlaAlaSerThrP 134

413 TCAGCCTGCTCTTCACTGTAGGGAGCGCTTGCCACCATGGTGCGGCCG 462
|||||||||||||||||||||||||||||||||||||||||||||||||||
135 heSerLeuLeuPheThrValGlyGluArgPheAlaThrMetValArgPro 150

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Figure 3A (cont.)

463 GTGGCCGAGAGCGGGGCCACCAAGACCAGCCGCGTCTACGGCTTCATCGG 512  
 |||||||  
 151 ValAlaGluSerGlyAlaThrLysThrSerArgValTyrGlyPheIleG1 167

513 CCTCTGCTGGCTGCTGGCCGCGCTGGGATGCTGCCTTGCTGGC 562  
 |||||||  
 168 yLeuCysTrpLeuLeuAlaAlaLeuLeuGlyMetLeuProLeuLeuGlyT 184

563 GGAACTGCCTGTGCGCCTTGACCGCTGCTCCAGCCTCTGCCCTCTAC 612  
 |||||||  
 185 rpAsnCysLeuCysAlaPheAspArgCysSerSerLeuLeuProLeuTyr 200

613 TCCAAGCGCTACATCCTCTTGCCTGGTATCTCGCCGGCGCCTGGC 662  
 |||||||  
 201 SerLysArgTyrIleLeuPheCysLeuValIlePheAlaGlyValLeuAl 217

663 CACCATCATGGGCCTCTATGGGCCATCTCCGCCTGGTGCAGGCCAGCG 712  
 |||||||  
 218 aThrIleMetGlyLeuTyrGlyAlaIlePheArgLeuValGlnAlaSerG 234

713 GGCAGAAGGCCAACGCCAGCGCCAGCGCCGCCGCAAGGCCGCCCTGCTG 762  
 |||||||  
 235 lyGlnLysAlaProArgProAlaAlaArgArgLysAlaArgArgLeuLeu 250

763 AAGACGGTGCTGATGATCCTGCTGGCCTCCTGGTGTGCTGGGCCACT 812  
 |||||||  
 251 LysThrValLeuMetIleLeuLeuAlaPheLeuValCysTrpGlyProLe 267

813 CTTCGGGCTGCTGGCGACGTCTTGGCTCCAACCTCTGGGCCAGG 862  
 |||||||  
 268 uPheGlyLeuLeuLeuAlaAspValPheGlySerAsnLeuTrpAlaGlnG 284

863 AGTACCTGCGGGCATGGACTGGATCCTGGCCCTGGCCGTCCTCAACTCG 912  
 |||||||  
 285 luTyrLeuArgGlyMetAspTrpIleLeuAlaLeuAlaValLeuAsnSer 300

913 GCGGTCAACCCCACATCTACTCCTCCGAGCAGGGAGGTGTGCAGAGC 962  
 |||||||  
 301 AlaValAsnProIleIleTyrSerPheArgSerArgGluValCysArgAl 317

Figure 3A (cont.)

963 CGTGCTCAGCTTCCTCTGCTGCGGGTGTCCTCGGCTGGCATGCGAGGGC 1012

|||||||||||||||||||||||||||||||||||||||||||||||||||||||

318 aValLeuSerPheLeuCysCysGlyCysLeuArgLeuGlyMetArgGlyP 334

1013 CCGGGGACTGCCTGGCCCGGCCGTCGAGGCTCACTCCGGAGCTTCCACC 1062

|||||||||||||||||||||||||||||||||||||||||||||||||||||||

335 roGlyAspCysLeuAlaArgAlaValGluAlaHisSerGlyAlaSerThr 350

1063 ACCGACAGCTCTTGAGGCCAAGGGACAGCTTCGCGGCTCCGCTCGCT 1112

|||||||||||||||||||||||||||||||||||||||||||||||||||

351 ThrAspSerSerLeuArgProArgAspSerPheArgGlySerArgSerLe 367

1113 CAGCTTCGGATGCGGGAGCCCCTGTCCAGCAGCTCCAGCGTGCGGAGCA 1162

|||||||||||||||||||||||||||||||||||||||||||||||||||

368 uSerPheArgMetArgGluProLeuSerSerSerSerValArgSerI 384

1163 TC 1164

||

385 le 385

Figure 3B

Comparison of HEDG7#M10 with HEDG7 polypeptide predicted from the genomic DNA sequence. Two amino acid substitutions relative to HEDG7 encoded by genomic DNA are found at positions 140 and 378 (bold, underlined).

1	50
hedg7#m10	MNATGTPVAP ESCQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR
huegd7	MNATGTPVAP ESCQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR
51	100
hedg7#m10	GLSVAASCLV VLENLLVLAA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL
huegd7	GLSVAASCLV VLENLLVLAA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL
101	150
hedg7#m10	ANVLLSGART FRLAPAQWFL REGLLFTALA ASTFSLLFTY GERFATMVRP
huegd7	ANVLLSGART FRLAPAQWFL REGLLFTALA ASTFSLLFTA GERFATMVRP
151	200
hedg7#m10	VAESGATKTS RVYGFIGLCW LLAALLGMLP LLGNCLCAF DRCSSLLPLY
huegd7	VAESGATKTS RVYGFIGLCW LLAALLGMLP LLGNCLCAF DRCSSLLPLY
201	250
hedg7#m10	SKRYILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL
huegd7	SKRYILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL
251	300
hedg7#m10	KTVLMI <del>L</del> AF LVCWGPLFGL LLADVF <del>G</del> SNL WAQEYLRGMD WILALAVLNS
huegd7	KTVLMI <del>L</del> AF LVCWGPLFGL LLADVF <del>G</del> SNL WAQEYLRGMD WILALAVLNS
301	350
hedg7#m10	AVNPIIYSFR SREVCRAVLS FLCCGCLRLG MRGPGDCLR AVEAHSGAST
huegd7	AVNPIIYSFR SREVCRAVLS FLCCGCLRLG MRGPGDCLR AVEAHSGAST
351	385
hedg7#m10	TDSSLRPRDS FRGSRSLSFR MREPLSSSS VRSI-
huegd7	TDSSLRPRDS FRGSRSLSFR MREPLSSIIS VRSI-

Figure 4

Multiple alignment of HEDG7 amino acid sequence with other edg family polypeptides, using the PILEUP (Wisconsin Package 9.0, Genetics Computer Group [GCG], Madison, Wisconsin) algorithm.

1	50
Edg-2	MAAISTSIPV ISQPQFTAMN EPQCFYNESI AFFYNRSGKH LATEWN.TVS
Edg-5	----- MNECHYDKHM DFFYNRSNTD TVDDWTGTKL
Edg-1	-----MGPTS VPLVKAHRSS VSDYVNYDII VRHYNYTGKL .NISADKENS
Edg-3	----- --MATALPPR LQPVRGNETL REHYQYVGKL AGRLEKEASEG
Edg-4	----- -----MGSL YSEYLNPNKV QEHYNYTKE. .TLETQETTS
Edg-7	-----MNATG TPVAPESCQQ LAAGGHSRLI VLHYNHSGRL AGRGGPEDGG
51	100
Edg-2	K.LVMGLGIT VCIFIMLANL LVMVAIYVNR RFHFPIYYLM ANLAAADFFA
Edg-5	V.IVLCVGT FCLFIFFSNS LVIAAVIKNR KFHFPFYLL ANLAAADFFA
Edg-1	IKLTSVVFIL ICCFIILENI FVLLTIWKT KFHRPMYYFI GNLALSDLLA
Edg-3	STLTTVLFLV ICSFIVLENL MVLIAIWKN KFHNRMYFFI GNLALCDLLA
Edg-4	RQVASAFIVI LCCAIIVVENL LVLIavarNS KFHSAMYLF L GNLAASDLLA
Edg-7	LGALRGLSVA ASCLVVLENL LVLAITSHM RSRRWVYYCL VNITLSDLLT
101	150
Edg-2	GLAYFYLMFN TGPNTTRRLTV STWLLRQGLI DTSLTASVAN LLAIAIERHI
Edg-5	GIAYVFLMFN TGPVSKTLTV NRWFRLRQGLL DSSLTASLTN LLVIAVERHM
Edg-1	GVAYTANLLL SGATTYKLTP AQWFLREGSM FVALSASVFS LLAIAIERYI
Edg-3	GIAYKVNILM SGKKTFSLSP TVWFLREGSM FVALGASTCS LLAIAIERHL
Edg-4	GVAFVANTLL SGSVTLRLTP VQWFAREGSA FITLSASVFS LLAIAIERHV
Edg-7	GAAYLANVLL SGARTFRLAP AQWFLREGLL FTALAASTFS LLFTVGERFA
151	200
Edg-2	TVFR.MQLHT RMSNRRVVVV IVVIWTMAIV MGAIPSVGWN CICDIENCSN
Edg-5	SIMR.MRVHS NLTKKRVTLL ILLVWAIAIF MGAVPTLGN CLCNISACSS
Edg-1	TMLK.MKLHN GSNNFRLFLL ISACWVISLI LGGLPIMGWN CISALSSCST
Edg-3	TMIK.MRPYD ANKRHRVFLL IGMCWLIAFT LGALPILGWN CLHNLPDCCST
Edg-4	AIAK.VKLYG SDKSCRMILL IGAWSLISLV LGGLPILGWN CLGHLEACST
Edg-7	TMVRPVAESG ATKTSRVYGF IGLCWLLAAL LGMLPLLGN CLCAFDRCSS

Figure 4 (cont.)

201	250
Edg-2 MAPLYSDSYL VFWAIFNLVT FVVMVVLYAH IFGYVRQRTM RMSRHSSGPR	
Edg-5 LAPIYSRSYLVFWTVSNLMA FLIMVVVYLR IYVYVKRKTN VLSPHTSGSI	
Edg-1 VLPLYHKHYI LFCTTVFTLL LLSIVILYCR IYSLVRTRSR RLTFRKNISK	
Edg-3 ILPLYSKKYI AFCISIFTAI LVTIVILYAR IYFLVKSSSR KVANHNN...	
Edg-4 VLPLYAKHYV LCVVTIFSII LLAVVALYVR IYCVVRSSHA DMA.....	
Edg-7 LLPLYSKRYI LFCLVIFAGV LATIMGLYGA IFRLVQASGQ KAPRPAARRK	
251	300
Edg-2 RNR.DTMMSL LKTVVIVLGA FIICWTPGLV LLLL.D.VCCP ..QCDVLAYE	
Edg-5 SRR.RTPMKL MKTVMTVLGA FVVCWTPGLV VLLLDGLNCR ..QCGVQHVK	
Edg-1 ASRSSENV AL LKTVIIVLSV FIACWAPLFI LLLL DV.GCK VKTC DILFRA	
Edg-3 ...SERSMAL LRTVVIVVSV FIACWSPLFI LFLIDV.ACR VQACPILFKA	
Edg-4 ...APQTLAL LKTVTIVLGV FIVCWLPAFS ILLLDY.ACP VHSCPILYKA	
Edg-7 ARR.....L LKTVL MILLA FLVCWGPLFG LLLADVFGSN LWAQEYLRGM	
301	350
Edg-2 KFFLLLAEFN SAMNPPIIYSY RDKE MSATFR QILCCQRSEN PTGPTESSDR	
Edg-5 RWFLLLALLN SVVNPIIYSY KDED MYGTMK KMICCF SQEN PERRPSRIPS	
Edg-1 EYFLVLAVLN SG TNPIIYTL TNKEMRRAFI RIMSC.CKCP SGDSAGKFKR	
Edg-3 QWFIVLAVLN SAMNPVIYTL ASKEMRRAFF RLVCN.CLVR GRGARASPIQ	
Edg-4 HYLFAVSTLN SLLNPVIYTW RSRDLRREVL RPL.Q.CWRP GVGVQGR.RR	
Edg-7 DWILALAVLN SAVNPIIYSF RSREV CRAVL SFLCCGCLRL GMRGPGDCLA	
351	399
Edg-2 SASSLNHTIL AGVHSNDHSV V----- ----- -----	
Edg-5 TVLSRS DTGS QYIEDSISQG AVCNKSTS-- ----- -----	
Edg-1 PIIAGMEFSR SKSDN NSSHPQ KDEGDN PETI MSSGNVN SSSS -----	
Edg-3 PALDPSRSKS SSSN NSSHSP KVKE DL PHTD PSSCIMDKNA ALQNGIFCN	
Edg-4 GGTPGHHL LP LRSSSSLERG MHMPTSPTFL EGNTVV-----	
Edg-7 RAVEAHSGAS TTDSSLR PRD SFRGSRSLSF RMREPLSSSS SVRSI-----	

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## FIGURE 5

Rat edg-7 partial genomic DNA sequence.

1 CCGTGTGTAT GGCTGCATCG GTCTGTGCTG GCTGCTGGCA GCTACCCCTGG  
51 GCCTGCTGCC CCTGCTGGGC TGGAACTGTG TGTGCGCCTT CCAGCGCTGC  
101 TCTAGCCTGC TGCCCCCTCTA CTCCAAGGGC TATGtGCTCT TTTGTGTGGT  
151 GGTCTTCGCC CTAATCCTAG TGACTATCCT GAGCCTCTAC GGGGCCATCT  
201 TTAGGGTGGT CCGAGCCAAC GGGCAGAAGT CCCCAGCTCC TCCTGCCCGC  
251 CGCAAGTCCC GCAGGCTACT CAACACCGTG CTGATGATCT TGGTGGCTTT  
301 TGTGGTGTGC TGGGGTCCCC TGTTTGGCCT GCTCCTGGCC GACATCTTG  
351 GATCTAAATGT CTGGGGCCAG GAGTACCTGC GCGGCATGGA CTGGATCCTG  
401 GCCCTAGCTG TGCTCAACTC AGCCATCAAT CCTCTCATCT ATTCCCTTCCG  
451 CAGCCGTGAG GTGCAGCAGC CTGTGCTGAC CTTCCGTGTC TGCAGCTGCC  
501 TCAGGTTAGG CCTGAGAGGC CctGGAgACT GCCTGACCCG GATCACCGAG  
551 GCCcACTcTG GGGCATCCAC CACTGACAGC TCGCTgAGGC CcAGGGAAAG  
601 TTTTCGGAct TCGAGGTAC TCaGCTTCAA gAtGCGAgAg CCGCTGTCCA  
651 GCGTTTCCAG CATCCCAGCG CCTAGAGCTT GAAcCAGCCG GTCGCCACC  
701 GAGCAGGCCT CCCAGGAAAA GTTAARAAGG ACTGGAMACA AGATCTYAGC  
751 CGACAGTGAY TGARAAATGC TTGCAGGCC CGGGTTCYTT CCACGAAYT  
801 CCCCCATGATG AATGTTYGGC AGGRAKKGCC AGATCCAGAT CCAGTGAGTC  
851 TGGGCCTCGA TGGGGCTCCC AGGCAGCAAA GGGGGTKTCC ATKTCGAGG  
901 CCATGGACGG GACAGGGCCT TACGGYTATT TCTTAGACAC AHKTKTCTG  
951 CKACCAGGAY GCTGYAACAT GTCTCTTGGT CACAGTGCTT TGGGGGTGTG  
1001 TCACTGGCAC ACAGTGCTTC GGGAGTGTGC TGGGAWGGGG TACACCTGCA  
1051 CCATTTGTTT GAAGACAACC WGAHGYGTYG TAAGAACTAC AGGAGGGGCT  
1101 GGGGGCACCC CAGTCTGTCA TCCATTCTC TTCTCAGTGA CTTCCCCAKT  
1151 GGGACAAGCA ACCTGCCCCC ATGGCCTCTC TCCTCCGGGT TCTCTATCTC  
1201 TCTGTGGGA GATAGACCCA CCCACCCGAG GTCTGGGCA ATCTCAACTG  
1251 GTCATGTAAC CCTACAGCCT CGCCCTTCCG GTTMTGAATC ACCAAGATAT  
1301 GCTGYGACAG GAAGCTGTGG ACTCXTACCT YGTGACAGTA CAG

**FIGURE 6****Rat edg-7 partial amino acid sequence.**

TRANSLATE of: redg7.seq check: 1095 from: 2 to: 673  
generated symbols 1 to: 224.

redg7.pep Length: 224 December 19, 1997 17:07 Type: P Check: 1302 ..

1 RVYGCIGLCW LLAATLGLLP LLGWNVCACF QRCSSLLPLY SKGYVLFCVV  
51 VFALILVTL SLYGAIFRVV RANGQKSPRP PARRKSRRLL NTVLMILVAF  
101 VVCWGPLFGL LLADIFGSNV WAQEYLRGMD WILALAVLNS AINPLIYSFR  
151 SREVQHAVLT FLCCGCLRLG LRGPGDCLTR ITEAHSGAST TDSSLRPRES  
201 FRTSRSLSFK MREPLSSVSS IPAP

Figure 7

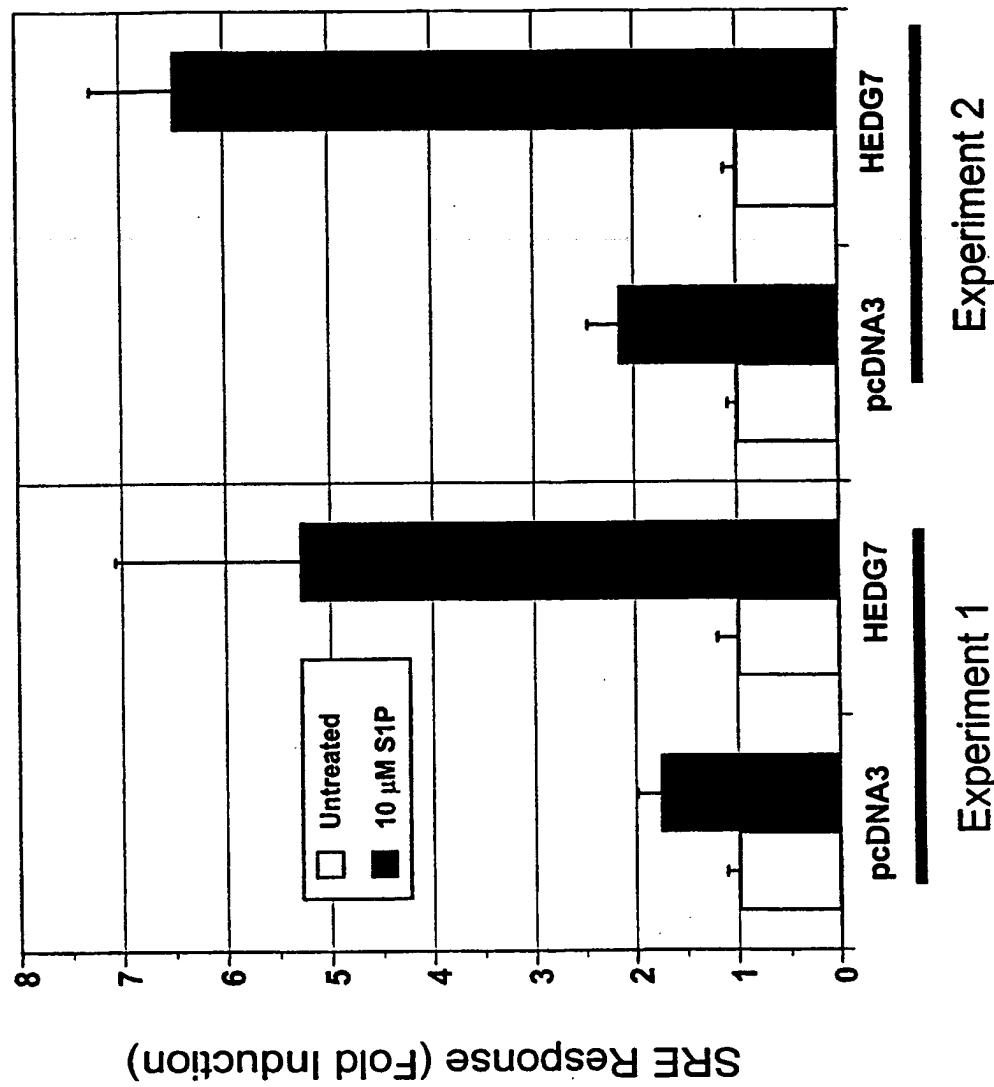
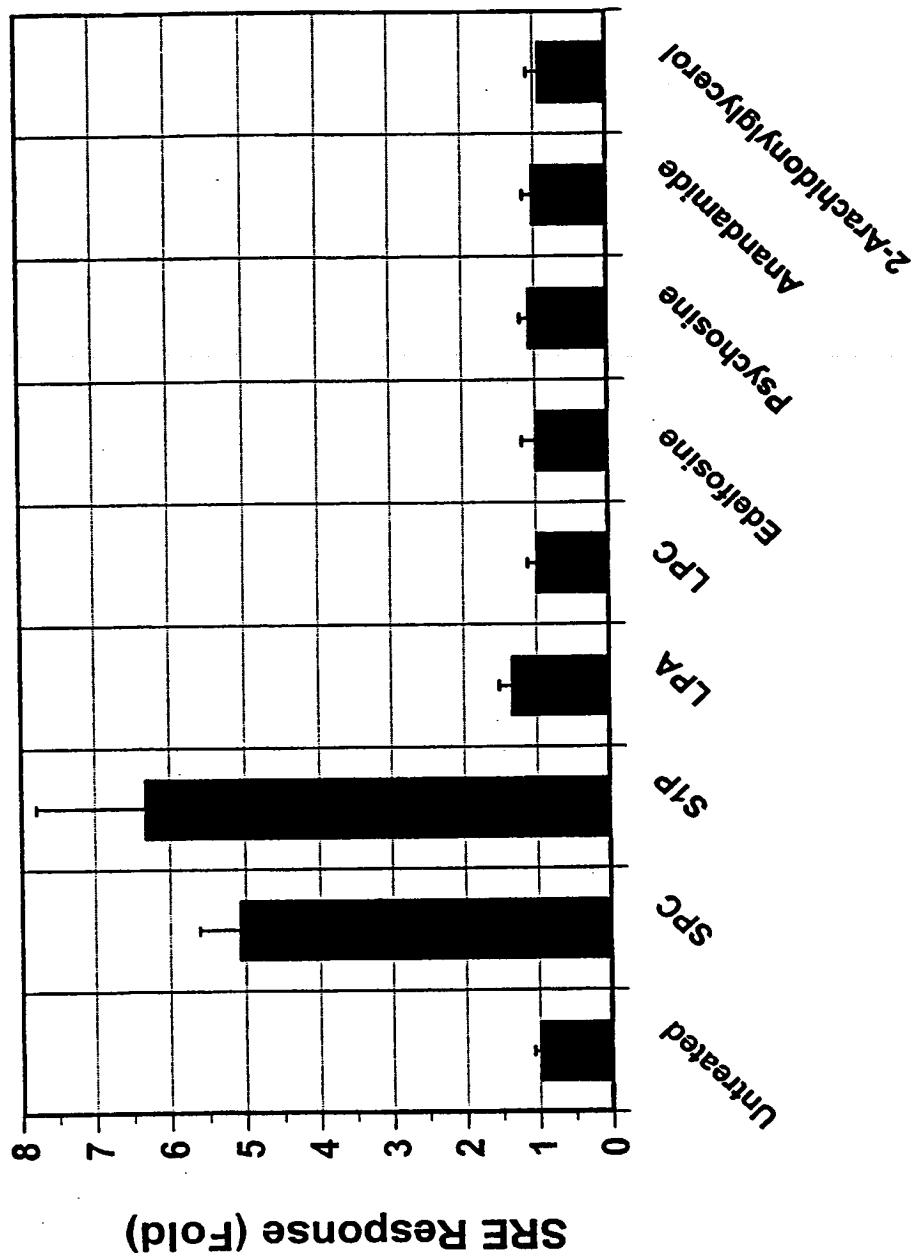


Figure 8



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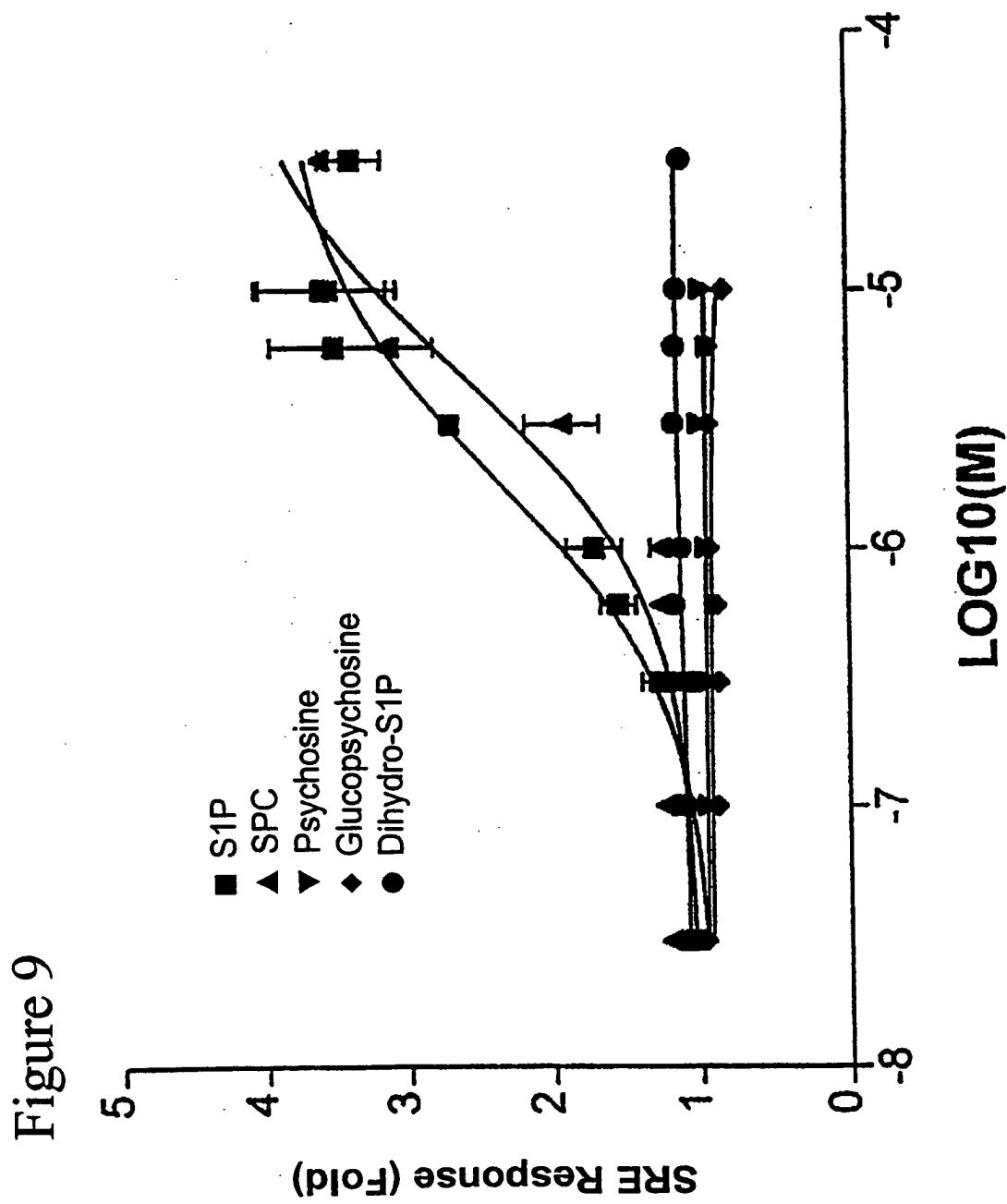


Figure 9

16/16

SEQUENCE LISTINGS

1

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

NAME: ALLELIX BIOPHARMACEUTICALS INC.  
STREET: 6850 Goreway Drive  
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PROVINCE: Ontario  
COUNTRY: Canada  
POSTAL CODE: L4V 1V7  
TELEPHONE: (905) 677-0831  
FACSIMILE: (905) 677-9595

## (ii) TITLE OF INVENTION: MAMMALIAN EDG-7 RECEPTOR HOMOLOGS

## (iii) NUMBER OF SEQUENCES: 4

## (iv) CORRESPONDENCE ADDRESS:

(A) NAME: Orange & Associates  
(B) STREET: Suite 3600, P.O. Box 190  
            Toronto Dominion Bank Tower  
            Toronto-Dominion Centre  
(C) CITY: Toronto  
(D) PROVINCE: Ontario  
(E) COUNTRY: Canada  
(F) ZIP: M5K 1H6

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: MS-DOS  
(D) SOFTWARE: Dos Editor

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE: 29-DEC-1998  
(C) CLASSIFICATION:

## (vi) PRIOR APPLICATION DATA:

(A) COUNTRY: U.S.A.  
(B) APPLICATION NUMBER: 60/070,184  
(C) FILING DATE: 30-DEC-1997  
(D) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Santosh K. Chari  
(B) FIRM: Orange & Associates  
(C) REFERENCE NUMBER: 8700213-0007  
(D) TELEPHONE: (416) 868-3457  
(E) FACSIMILE: (416) 364-7910

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2270 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

1   CCCCGGGG AGGCCATGAA CGCACGGGG ACCCCGGTGG CCCCCGAGTC  
51   CTGCCAACAG CTGGCGGCCG GCGGGCACAG CCGGCTCATT GTTCTGCACT  
101   ACAACCACTC GGGCCGGCTG GCCGGGCGCG GGGGGCCGGA GGATGGCGGC  
151   CTGGGGCCC TGCGGGGCT GTCGGTGGCC GCCAGCTGCC TGGTGGTGCT  
201   GGAGAACTTG CTGGTGCTGG CGGCCATCAC CAGCCACATG CGGTCGCGAC  
251   GCTGGGTCTA CTATTGCCCTG GTGAACATCA CGCTGAGTGA CCTGCTCACG  
301   GGCGCGGCCCT ACCTGGCCAA CGTGCTGCTG TCGGGGGCC GCACCTTCCG  
351   TCTGGCGCCC GCCCAGTGGT TCCTACGGGA GGGCCTGCTC TTCACCGCCC  
401   TGGCCGCCTC CACTTTCAGC CTGCTCTTCA CTGCAGGGGA GCGCTTTGCC  
451   ACCATGGTGC GGCGGGTGGC CGAGAGCGGG GCCACCAAGA CCAGCCGCGT  
501   CTACGGCTTC ATCGGCCTCT GCTGGCTGCT GGCGCGCTG CTGGGGATGC  
551   TGCCTTGCT GGGCTGGAAC TGCCTGTGCG CCTTGACCG CTGCTCCAGC  
601   CTTCTGCCCT TCTACTCCAA GCGCTACATC CTCTTCTGCC TGGTGATCTT  
651   CGCCGGCGTC CTGGCCACCA TCATGGGCCT CTATGGGCC ATCTTCCGCC  
701   TGGTGCAGGC CAGCGGGCAG AAGGCCCCAC GCCCAGCGGC CCGCCGCAAG  
751   GCCCGCCGCC TGCTGAAGAC GGTGCTGATG ATCCTGCTGG CCTTCCTGGT  
801   GTGCTGGGGC CCACTCTTCG GGCTGCTGCT GGCGACGTC TTTGGCTCCA  
851   ACCTCTGGGC CCAGGAGTAC CTGCGGGCA TGGACTGGAT CCTGGCCCTG  
901   GCCGTCCCTCA ACTCGGCCGT CAACCCCATC ATCTACTCCT TCCGCAGCAG  
951   GGAGGTGTGC AGAGCCGTGC TCAGCTTCCT CTGCTGCCGG TGTCTCCGGC  
1001   TGGGCATGCG AGGGCCCGGG GACTGCCTGG CCCGGGCCGT CGAGGCTCAC  
1051   TCCGGAGCTT CCACCACCGA CAGCTCTCTG AGGCCAAGGG ACAGCTTTCG  
1101   CGGCTCCCGC TCGCTCAGCT TTGGATGCG GGAGCCCTG TCCAGCATCT  
1151   CCAGCGTGCG GAGCATTGA AGTTGCAGTC TTGCGTGTGG ATGGTGCAGC  
1201   CACCGGGTGC GTGCCAGGCA GGCCCTCCTG GGGTACAGGA AGCTGTGTGC  
1251   ACGCAGCCTC GCCTGTATGG GGAGCAGGGA ACGGGACAGG CCCCCATGGT  
1301   CTTCCCGGTG GCCTCTCGGG GCTTCTGACG CCAAATGGC TTCCCATGGT  
1351   CACCCCTGGAC AAGGAGGCAA CCACCCACC TCCCCGTAGG AGCAGAGAGC  
1401   ACCCTGGTGT GGGGGCGAGT GGGTTCCCCA CAACCCGCT TCTGTGTGAT  
1451   TCTGGGAAG TCCCGGCCCTG TCTCTGGGCC TCAGTAGGGC TCCCAGGCTG  
1501   CAAGGGGTGG ACTGTGGGAT GCATGCCCTG GCAACATTGA AGTTCGATCA  
1551   TGGTACGTGA TGTTGCAGGCC TCTTATTCCC TGGTGCAGTGC ATGTGTGGGG

1601 GCCGTGGCTC AGGGGGGCTG TGGATCTAGG GGCAGCCGGG TGTGTCTTG  
 1651 CTAGAGAGGG CCACGGGCCA GTGCCCTGTG AGGGTGGAGA GTGTGTGTGT  
 1701 GTGTGTGTGT GTGTGTGTGG ACAACYTCTG GGCGTTGCCGG GAAGTGGGGG  
 1751 TGACAATGAC AGTTAATGCC GCTCTTCTTG TTCACTTCAC CTTTAGAAAT  
 1801 GGCAGGGCCC ATGCCCATC TCTGGCYTCT GCATCTTTG GGGACCCACT  
 1851 CTCTGGGGCT GGCAGAGGCA CCACCTTGGC TTCTGGGCT GGGGGAATCT  
 1901 TCCCTCACAT CCCCTTCAGC ATGAACGGCC TCGGCTTCC CGGTGGGTAA  
 1951 AACAGTTAA TCAGTGAAGC CGAACGACAG GGTTGATGGT ACACGCTCCC  
 2001 CGCCAGCCAC AGGGGCTGAC GACTGCCTGC CCCGTGAAAC TCCAGTGGAG  
 2051 ACGTTTCAGC TCCACACCCT TCAGTATGGG AGACGCCAGC CCCACGGGC  
 2101 TACGGTGCAA GCAGATAACT GAATTTCGAA GTGTAGGTTG TGTTTAATT  
 2151 GAATCTGTTT ATATTTCGGT AGCCCCATGG GGCGGGTGGG GGGGATCCAC  
 2201 TAGTTCTAGA GCGGCCGCCA CCGCGGTGGA GCTCCAGYWT TWGWTCCCKT  
 2251 TAGTGAGGGT TAATTGCGCG

2270

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1176 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

1 AAGCTTGCCA CCATGAACGC CACGGGGACC CCGGTGGCCC CCGAGTCCTG  
 51 CCAACAGCTG GCGGCCGGCG GGCACAGCCG GTCATTGTT CTGCACTACA  
 101 ACCACTCGGG CCGGCTGGCC GGGCGCGGGG GGCCGGAGGA TGGCGGCCTG  
 151 GGGGCCCTGC GGGGGCTGTC GGTGGCCGCC AGCTGCCTGG TGTTGCTGGA  
 201 GAACTTGCTG GTGCTGGCGG CCATCACCAAG CCACATGCCG TCGCGACGCT  
 251 GGGTCTACTA TTGCCTGGTG AACATCACGC TGAGTGACCT GCTCACGGGC  
 301 GCGGCCTTACCG TGCCCAACGT GCTGCTGTG GGGGCCCGCA CCTTCCGTCT  
 351 GGCGCCCGCC CAGTGGTTCC TACGGGAGGG CCTGCTCTTC ACCGCCCTGG  
 401 CCGCCTCCAC CTTCAGCCTG CTCTTCACTG TAGGGGAGCG CTTTGCACC  
 451 ATGGTGCAGGC CGGTGGCCGA GAGCGGGGCC ACCAAGACCA GCCGCGTCTA  
 501 CGGCTTCATC GGCTCTGCT GGCTGCTGGC CGCGCTGCTG GGGATGCTGC  
 551 CTTTGCTGGG CTGGAACTGC CTGTGCGCCT TTGACCGCTG CTCCAGCCTT  
 601 CTGCCCCCTCT ACTCCAAGCG CTACATCCTC TTCTGCCTGG TGATCTTCGC  
 651 CGGCGTCTG GCCACCATCA TGGGCCTCTA TGGGGCCATC TTCCGCCTGG  
 701 TGCAGGCCAG CGGGCAGAAG GCCCCACGCC CAGCGGCCCG CCGCAAGGCC  
 751 CGCCGCCTGC TGAAGACGGT GCTGATGATC CTGCTGGCCT TCCTGGTGTG

801 CTGGGGCCA CTCTCGGGC TGCTGCTGGC CGACGTCTTT GGCTCCAACC  
851 TCTGGGCCA GGAGTACCTG CGGGGCATGG ACTGGATCCT GGCCCTGGCC  
901 GTCCTCAACT CGGCAGGTCAA CCCCATCATC TACTCCTTCC GCAGCAGGGA  
951 GGTGTGCAGA GCCGTGCTCA GCTTCCTCTG CTGCGGGTGT CTCCGGCTGG  
1001 GCATGCGAGG GCCCGGGGAC TGCCTGGCCC GGGCCGTCGA GGCTCACTCC  
1051 GGAGCTTCCA CCACCGACAG CTCTCTGAGG CCAAGGGACA GCTTTCGCGG  
1101 CTCCCGCTCG CTCAGCTTTC GGATGCCGGGA GCCCCTGTCC AGCAGCTCCA  
1151 GCGTGCGGAG CATCTGAAGT TCTAGA

1176

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 384 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

1 MNATGTPVAP ESCQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR  
51 GLSVAASCLV VLENLLVLAA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL  
101 ANVLLSGART FRLAPAQWFL REQLLFTALA ASTFSLLFTA GERFATMVRP  
151 VAESGATKTS RVYGFIGLCW LLAALLGMLP LLGWNCLCAF DRCSSLLPLY  
201 SKRYILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL  
251 KTVLMILLAF LVCWGPIFGL LLADVFGSNL WAQEYLRGMD WILALAVLNS  
301 AVNPIIYSFR SREVCRAVLS FLCCGCLR LG MRGP GDCLAR AVEAHSGAST  
351 TDSSLRPRDS FRGSRSLSFR MREPLSSIIS VRSI

384

## (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 384 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

1 MNATGTPVAP ESCQQLAAGG HSRLIVLHYN HSGRLAGRGG PEDGGLGALR  
51 GLSVAASCLV VLENLLVLA ITSHMRSRRW VYYCLVNITL SDLLTGAAYL  
101 ANVLLSGART FRLAPAQWFL REGLLFTALA ASTFSLLFTV GERFATMVRP  
151 VAESEGATKTS RVYGFIGLCW LLAALLGMLP LLGWNCLCAF DRCSSLLPLY  
201 SKRYILFCLV IFAGVLATIM GLYGAIFRLV QASGQKAPRP AARRKARRLL  
251 KTVLMILLAF LVCWGPLFGL LLADVFGSNL WAQEYLRGMD WILALAVLNS  
301 AVNPIIYSFR SREVCRAVLS FLCCGCLRLG MRGPGDCLR AVEAHSGAST  
351 TDSSLRPRDS FRGSRSLSFR MREPLSSSS VRSI

384



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/12, C07K 14/705, C12N 15/85, 5/10, C12Q 1/68, G01N 33/68</b>		A3	(11) International Publication Number: <b>WO 99/35106</b> (43) International Publication Date: <b>15 July 1999 (15.07.99)</b>
<p>(21) International Application Number: <b>PCT/CA98/01196</b></p> <p>(22) International Filing Date: <b>30 December 1998 (30.12.98)</b></p> <p>(30) Priority Data: <b>60/070,184 30 December 1997 (30.12.97) US</b></p> <p>(71) Applicant (<i>for all designated States except US</i>): <b>ALLELIX BIOPHARMACEUTICALS INC. [CA/CA]; 6850 Goreway Drive, Mississauga, Ontario L4V 1V7 (CA).</b></p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): <b>MUNROE, Donald, G. [CA/CA]; 27 Wakefield Lane, Waterdown, Ontario L0R 2H3 (CA). GUPTA, Ashwani, K. [CA/CA]; 7031 Dunrobin Way, Mississauga, Ontario L5N 6Y4 (CA). ZASTAWNÝ, Roman, L. [CA/CA]; 21 Newstead Road, Etobicoke, Ontario M9P 3G2 (CA).</b></p> <p>(74) Agents: <b>CHARI, Santosh, K. et al.; Orange &amp; Associates, Suite 3600, Toronto Dominion Bank Tower, Toronto-Dominion Centre, P.O. Box 190, Toronto, Ontario M5K 1H6 (CA).</b></p>		<p>(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</b></p> <p><b>Published</b> <i>With international search report.</i></p> <p>(88) Date of publication of the international search report: <b>16 September 1999 (16.09.99)</b></p>	

(54) Title: **MAMMALIAN EDG-7 RECEPTOR HOMOLOGS**

## (57) Abstract

The present invention is directed to nucleic acid sequence and amino acid sequences for mammalian EDG-7 receptor homologs, and particularly for human EDG-7 receptor homologs. The invention also provides methods for determining agonists and antagonists for EDG-7 receptors in addition to assays, expression vectors, host cells and methods for treating disorders associated with aberrant expression or activity of EDG-7.

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CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Republik of Korea	RO	Romania		
CU	Cuba	LC	Kazakhstan	RU	Russian Federation		
CZ	Czech Republic	LI	Saint Lucia	SD	Sudan		
DE	Germany	LK	Liechtenstein	SE	Sweden		
DK	Denmark	LR	Sri Lanka	SG	Singapore		
EE	Estonia		Liberia				

# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/CA 98/01196

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6	C12N15/12	C07K14/705	C12N15/85	C12N5/10	C12Q1/68
G01N33/68					

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SONGZHU AN ET AL.: "Molecular cloning of the human Edg2 protein and its identification as a functional cellular receptor for lysophosphatidic acid"            BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 231, 24 February 1997, pages 619-622,            XP002046899            ORLANDO, FL US            see the whole document</p> <p>---</p> <p>WO 97 00952 A (INCYTE PHARMACEUTICALS, INC.) 9 January 1997            see page 4, line 28 - page 9, line 23;            claims; examples</p> <p>---</p> <p>---</p>	1-3, 15, 17
X		1, 2, 15, 17

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
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Date of the actual completion of the international search

29 June 1999

Date of mailing of the international search report

12/07/1999

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Authorized officer

Montero Lopez, B

# INTERNATIONAL SEARCH REPORT

Inte<sup>r</sup>na<sup>t</sup>ional Application No  
PCT/CA 98/01196

**C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>FUMINORI YAMAGUCHI ET AL.: "Molecular cloning of the novel human G protein-coupled receptor (GPCR) gene mapped on chromosome 9"  <b>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS</b>, vol. 227, no. 2, 14 October 1996, pages 608-614, XP002107530 ORLANDO, FL US  see abstract; figure 1  see page 612, paragraph 2 - page 613, paragraph 1  -----</p>	1, 2, 15, 17
P, X	<p>WO 98 50549 A (HUMAN GENOME SCIENCES, INC.) 12 November 1998  see page 3, line 11 - line 23  see page 3, line 26 - line 28  see page 4, line 2 - line 16  see page 5, line 4 - line 10  see page 5, line 28 - page 6, line 7  see page 7, line 30 - page 17, line 12  see page 17, line 28 - page 18, line 2;  figure 3; examples 5-7  -----</p>	1-15, 17
P, X	<p>WO 98 48016 A (INCYTE PHARMACEUTICALS, INC.) 29 October 1998  see page 2, line 15 - line 31  see page 9, line 26 - page 15, line 18  see page 21, line 25 - page 22, line 21  see page 30, line 23 - line 27  see page 33, line 31 - page 34, line 12;  examples I-IX  -----</p>	4-6, 8-15
A	<p>R.C. LEVITT ET AL.: "Mapping of the gene for hormone sensitive lipase (LIPE) to chromosome 19q13.1-q13.2"  <b>CYTOGENETICS AND CELL GENETICS</b>, vol. 69, 1995, pages 211-214, XP002107531 cited in the application  see figure 1  -----</p>	

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 98/01196

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: 16, 18 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
Claims directed to ligands and antagonists have not been searched due to the lack of adequate technical description thereof in the application
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No

PCT/CA 98/01196

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9700952	A 09-01-1997	AU CA EP	6388696 A 2224799 A 0840787 A		22-01-1997 09-01-1997 13-05-1998
WO 9850549	A 12-11-1998		NONE		
WO 9848016	A 29-10-1998	AU	7132898 A		13-11-1998

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